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The Use of a Derivative of Hematoporphyrin in Tumor Detection[1, 2, 3]

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SUMMARY

The localization of a hematoporphyrin derivative and its detection by fluorescence have been studied. It has been demonstrated that the derivative is a better localizer than the crude hematoporphyrin and that it has a greater tendency to accumulate in malignant than in nonmalignant tissue. In general, the greatest fluorescence is exhibited at the periphery of the tumor. By utilizing the acetic acid-sulfuric acid derivative of hematoporphyrin and a proper activating and viewing system, one can detect malignancy by fluorescence. This improved technique appears to be sufficiently more flexible than that used by earlier workers to warrant trial in human beings under certain circumstances.—J. Nat. Cancer Inst. 26: 1–11, 1961.

IT HAS been demonstrated that hematoporphyrin tends to accumulate in tumors (*1*, *2*) and that its red fluorescence might be utilized in the visualization and delineation of neoplastic tissue (*2–5*). Early studies in animals and human beings showed some promise; however, the procedures employed were unrefined and gave inconsistent results. These procedures also required large amounts of hematoporphyrin (*3–7*), which increased the danger of photosensitivity and at the same time tended to decrease the degree of specificity. Furthermore, it has been shown by Schwartz and co-workers (*8*, *9*) that the previously used hematoporphyrin was a crude mixture of many porphyrins, and that, in reality, it is the so-called impurity components that are the good localizers rather than the relatively pure hematoporphyrin which is a poor localizer.

Because of the foregoing, we felt that further investigation was warranted in an attempt to evaluate whether a more refined technique based on the accumulation of an administered porphyrin in a tumor could be utilized as a cancer-detection procedure under certain circumstances.

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[2] Abridgment of portion of thesis submitted by Dr. Lipson to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

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[5] The Mayo Foundation, Rochester, Minnesota, is a part of the Graduate School of the University of Minnesota.

To do this, we decided to employ one of the derivatives of hematoporphyrin hydrochloride in an effort to eliminate the effect that would result from the use of the crude preparations, which vary in composition. In addition, the activating wavelengths required to produce the fluorescence, as well as a method for detecting the fluorescence, were to be more adequately defined and more efficiently utilized.

MATERIALS AND METHODS

Preparation of hematoporphyrin derivative.—Recrystallized hematoporphyrin hydrochloride [6] was used. The crude hematoporphyrin was dissolved in a mixture of 19 parts of glacial acetic acid and 1 part concentrated sulfuric acid and allowed to stand for 15 minutes. The mixture was then filtered to remove the undissolved residue. Next, the porphyrin solution was neutralized by the addition of 15 to 20 volumes of an approximately 3 percent sodium acetate solution; Congo Red paper was used as the indicator. With neutralization, the hematoporphyrin derivative precipitated out and was removed by filtration. The precipitate was washed with distilled water several times and allowed to dry in the air, at room temperature, in the dark. The hematoporphyrin derivative was then dissolved in saline solution made alkaline by the addition of normal sodium hydroxide. The final *p*H was adjusted to 7.4 by the addition of hydrochloric acid. Solutions containing 10 mg per ml were used throughout the experimental studies. These were kept in brown bottles and refrigerated when not in use. Hereafter for purposes of brevity the hematoporphyrin derivative will be referred to for the most part as the "derivative."

Animals and tumors.—Four tumors were used for transplantation: a rhabdomyosarcoma, a mammary adenocarcinoma transplantable in C57BL mice, a Walker 256 carcinosarcoma, and a Flexner-Jobling carcinoma transplantable to the Sprague-Dawley rat.[7] The tumors were transplanted by subcutaneous injection of 0.2 cc of a tumor-cell suspension into an appropriate animal. Twenty to several hundred animals were used for each experiment.

Activation and detection of fluorescence.—The light source used by previous workers to activate the crude hematoporphyrin was a standard ultraviolet lamp with various filters. Because these lamps are inefficient in the production of a sufficiently intense amount of the activating wavelengths of the crude hematoporphyrin as well as the derivative, and because they also lend themselves poorly to optical manipulation, we decided to use a different light source and filter system.

A direct-current carbon arc lamp run at 150 volts and 10 amperes and utilizing cored carbons was used as the source of light. The light was

[6] Available commercially from the Nutritional Biochemicals Corp., Cleveland, Ohio.

[7] We are grateful to Mr. Kline of the Wisconsin Alumni Research Foundation who supplied us with the Walker and Flexner-Jobling tumors.

passed through water to cool it and then through a sufficient amount of copper sulfate solution which removed all the red light when combined with a Corning No. 759 glass filter (filter transmits more than 70% between 330 and 407 mμ). This filter system was used because it delivered an intense amount of the maximally activating wavelengths (400 to 407 mμ) of the derivative as determined by our earlier studies (*10*), and at the same time it did not transmit any red light as did the activating lights of other workers.

A Corning #368 glass filter, which did not transmit any activating light, was used for viewing the tumor fluorescence (630 mμ). Thus, only the red fluorescence and none of the activating light reached the eye. The same filter system and light source were used for photography with both color film and black-and-white film.

STUDIES

Localization of Hematoporphyrin Derivative

Animals and tumors of different ages and sizes were given varying amounts of the derivative by subcutaneous injection into an area far removed from the tumor, by intraperitoneal injection, or by tail vein. Fluorescence of the total animal as well as of the tumor was followed by the daily killing of ten animals from each tumor group. The animals were exposed to the light system for external examination, and then the skin over the tumor was flapped back so that the tumor could be examined. The study of a group was continued until the fluorescence disappeared or the group of animals was depleted.

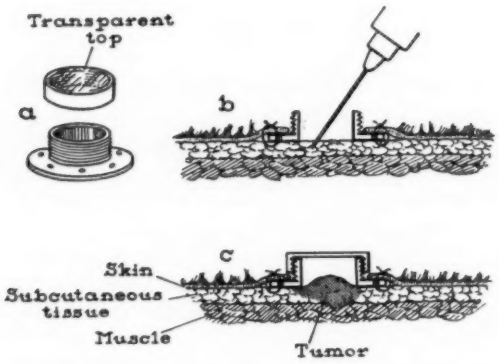
Results.—It was found that all tumors showed localization of the porphyrin as determined by fluorescence, and that the greatest intensity of fluorescence was localized at the site of invasion of normal tissue by the malignant tumor. The interior of the mammary adenocarcinoma and the rhabdomyosarcoma exhibited little-to-no fluorescence, whereas the interior of the Walker and the Flexner-Jobling tumors showed slight-to-moderate fluorescence. In all tumors the intensity of fluorescence of the interior appeared to increase with increased doses of derivative. Necrotic portions of the tumors were not fluorescent.

Fluorescence of all tumors lasted much longer than fluorescence of the intact animal, which appeared to vary directly with the amount of derivative received. When the animals were given small amounts of derivative, fluorescence of the tumor began to decrease within 48 hours and disappeared completely within 6 days; when larger amounts were given, the fluorescence lasted 14 days or more.

Fluorescence of the intact animal also depended on the amount of derivative administered and was greatest at the site of injection. When present elsewhere, however, the fluorescence was most prominent in the periorbital, perioral, and nasal regions, and to a lesser extent on the ears, the soles of the feet, and the tail.

Fluorescence exhibited in the internal organs depended on the dosage of derivative, on the time interval between giving the porphyrin and examination, and on the route of administration, with intraperitoneal injection causing diffuse staining of all the abdominal viscera. Usually the gallbladder showed considerable fluorescence, since the porphyrin is excreted in the bile.

Rapidity of localization of hematoporphyrin derivative.—A plastic cap with a screw top was implanted into the backs of the rats used in this study (text-fig. 1). The cap was anchored by subcutaneous sutures; the area directly beneath the cap was denuded, and the top was screwed into place. In half the animals a suspension of Walker carcinosarcoma cells was injected into this region; the rest of the animals did not receive a tumor transplant and were used as controls. When the tumors had



TEXT-FIGURE 1.—*a*. Plastic cap and transparent top. *b*. Diagrammatic view of plastic cap sutured in place and of injection of tumor cells into denuded subcutaneous tissue. *c*. Diagrammatic view of plastic cap with top in place and visible tumor.

reached a suitable size, both tumor-bearing and non-tumor-bearing animals were given a continuous intravenous infusion of derivative in a dose of 0.01 mg per g of body weight. This was diluted to a total of 1 cc with physiologic saline and run in over 6½ hours. Examination and color photography of the area were done just prior to the start of the infusion, at hourly intervals thereafter for 12 hours, and then at 16, 24, 36, 48, and 60 hours after the onset of the infusion. The tops of the caps were removed at the start of the experiment, and the caps were filled with saline between examinations and aspirated just before examination.

Results.—The tumors began to show fluorescence approximately 4 hours after the infusion was started. Fluorescence increased in intensity during the next few hours and reached a maximum in 7 to 8 hours, after which it leveled off. The control animals did not show any fluorescence until at least 8 hours after the infusion was started, probably because of the staining of the tissues and coagulum under the cap by the derivative

in the interstitial fluid that circulated in the area. The saline that had been instilled into the caps between examinations showed some fluorescence, as did the animals' urine both during the infusion and for several hours after it was completed.

Thus it was evident that a slow, continuous, intravenous infusion of the derivative allowed tumor differentiation by fluorescence, which reached maximal intensity in 7 to 8 hours after the start of the infusion. Two logical questions then arose: Will a single injection of the derivative allow a similar differentiation of the tumor by fluorescence? If so, can it be done with the utilization of a smaller dosage of the derivative as well as a shorter time interval between the administration of the derivative and examination? In an attempt to answer these questions the following studies were carried out.

Determination of the Minimal Single Differentiating Dose of Derivative

The object of this study was to determine the minimal amount of derivative given as a single injection required to obtain the best localization of the fluorescence at the tumor site only. All 4 tumors were used, and since the mammary and Flexner-Jobling carcinomas were slower growing than the other 2 tumors, the size of the tumor rather than its age was used as a means of selecting animals for comparison.

The animals were given the derivative by single subcutaneous or intravenous injections. Dosages of 0.1, 0.05, 0.008, and 0.002 mg per g of body weight were used. The animals were examined 6 to 8 hours after receiving the porphyrin because of the results of the previous experiment.

Results.—A dose of 0.05 mg per g of body weight was found to offer the best localization and differentiation of the tumor by fluorescence. The gallbladders of most animals were fluorescent, and in a few the esophageal and tracheal mucosa as well as the parenchyma of the lungs were slightly fluorescent.

Because of the occasional slight fluorescence of regions other than the tumor, a further attempt was made to confine the fluorescence entirely to the tumor area. To do this, a single intravenous injection of 0.002 mg of derivative per g of body weight was given to animals bearing the same tumor transplant that had been used previously. The animals were killed at hourly intervals for 6 hours after injection and were examined for fluorescence. In addition, however, we increased the intensity of the activating light by focusing the beam of light with a lens.

The animals showed moderate fluorescence confined entirely to the tumor 1 hour after administration of the derivative. The fluorescence increased in intensity during the next 2 hours and then appeared to level off. Thus, by coupling a small, single dose of the derivative with an increased amount of activating light, we were able to confine tumor differentiation by fluorescence entirely to the tumor. Furthermore, this

differentiation was possible within 3 hours after administration of the derivative.

Effect of Size and Age of Tumor on Localization of Derivative

Suspensions of all 4 tumors were injected subcutaneously into mice or rats. Twenty-four hours after tumor transplants examination was begun in animals that had been prepared 3 to 4 hours previously with hematoporphyrin derivative given by intraperitoneal injection in a dose of 0.002 mg per g of body weight. Examinations were carried out daily for 3 days and then weekly until the tumor killed the animal, which was usually in 6 to 8 weeks.

Results.—Localization and detection of tumors were possible 24 hours after transplantation and continued until the tumor became necrotic. Since suspensions of neoplastic cells were used in transplantation, no tumors were visible in white light 24 hours later. The presence of malignant cells in the portions that had become fluorescent was confirmed microscopically.

Localization in Nonmalignant Tissue

In an attempt to simulate benign tumefaction, polyvinyl sponges, talcum powder, and mineral oil were implanted subcutaneously in mice and rats. The animals were given 0.002 mg of derivative per g of body weight at varying time intervals after implantation and were examined for fluorescence 3 hours later. Also during the course of this study, benign mammary fibroadenomas occurred spontaneously in several mice and rats. One rat tumor was successfully transplanted to other animals and was utilized along with the other spontaneous tumors.

Results.—The polyvinyl sponges, talcum powder, and mineral oil produced foreign-body reactions which did not localize the porphyrin so that it could be detected by fluorescence. Only a few benign tumors, including the transplantable one, exhibited minimal localized fluorescence; however, fluorescence was never of the same intensity in benign tumors as it was in malignant tumors.

Comparison of Crude Hematoporphyrin With Hematoporphyrin Derivative

Animals with transplants of one of the 4 tumors were given either crude hematoporphyrin or derivative by intravenous or subcutaneous injection 3 hours before examination. The dose utilized was 0.002 mg per g of body weight. The animals were killed, and the tumors were exposed and examined for fluorescence.

Results.—The derivative produced good localized fluorescence in all 4 types of tumors, whereas the crude preparation gave adequate fluorescence in only the Flexner-Jobling and mammary carcinomas, but none in the rhabdomyosarcoma and Walker carcinosarcoma.

COMMENTS

Our studies clearly showed that, in mice and rats, hematoporphyrin derivative localized in malignant tissue which then appeared fluorescent on activation with an appropriate light, but small doses of crude hematoporphyrin gave inconsistent results.

The specificity of localization appeared to be greatest in malignant tissue. Accumulation of hematoporphyrin and its detection by fluorescence have been reported to occur in such tissues as lymphatic, embryonic, and traumatic tissue (*5*, *7*, *11*) as well as in areas of hyperplasia (*12*). Our studies indicated that the accumulation of the derivative and the rapidity of uptake were greatest in malignant tissue. Occasionally we observed minimal accumulation in lymphatic tissue, in a few benign fibroadenomas, and in some fresh wounds; however, we believe the fluorescence in the wounds was due to the porphyrin in the serum that had seeped into the area. We did not get significant accumulation in any granulomatous lesions, nor did we find that the derivative passed the placental barrier to the cord or fetus; however, the uterus and membranes did show fluorescence.

It is thus apparent that by adequately defining the wavelengths which maximally activate the derivative, we were able to use a small dose and still obtain excellent tumor fluorescence (630 mμ). We accomplished this by utilizing a sufficiently intense amount of the specific activating wavelengths of the derivative and then viewing the area in question through the proper filter, which facilitated the visual detection of fluorescence.

Naturally, one wonders how this localization and subsequent fluorescence can be used in human beings. We feel, on the basis of our studies, that this procedure has considerable clinical applicability which was not apparent before our study. Previous workers found that doses of 100 to 1000 mg of crude hematoporphyrin were required to obtain fluorescence of a tumor (*7*). As the crude material is converted to a particular derivative, a common denominator is provided for all batches of commercial hematoporphyrin. This derivative is a much better localizer than the crude material, and small doses give consistent results even in microscopic aggregates of malignant cells. The small amount of derivative required is easily handled by the body and is well within the range of safety. We also have studied the photosensitizing potential of the derivative, but have not found signs of photosensitivity in any animal which received this small amount of derivative, despite exposures to intense light for prolonged periods, as described in an earlier communication (*10*). By using a single intravenous injection rather than a prolonged continuous infusion, we feel that the derivative allows more flexibility. This short administration time, coupled with the relatively short time necessary between administration and examination, allows the entire procedure to be carried out in slightly more than 3 hours.

Because of the greater flexibility provided by the derivative and the improved activating and viewing systems, we are of the opinion that its trial in the human is indicated and, therefore, we have initiated a clinical

study to investigate fluorescent endoscopy as a diagnostic tool. Our results at the time of this writing are promising and will be reported in a future communication.

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PLATE

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PLATE

PLATE 1

FIGURE 1.—Rhabdomyosarcoma 3 hours after hematoporphyrin derivative administration. *Left* shows tumor in white light. *Right* shows red fluorescence of tumor as seen with the activating light plus filter system.

FIGURE 2.—Benign cystic tumor 3 hours after hematoporphyrin derivative administration. *Left* shows tumor in white light. *Right* shows no fluorescence of the tumor when viewed with activating light and filter system.

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Evaluation of Drug Resistance in Cell Cultures by Differential Toxicity Tests [1,2]

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SUMMARY

Drug-resistant lines were obtained by cyclic exposure of clone strains of pig kidney cells to aminopterin, 2,6-diaminopurine, and allylglycine. Cultures of sensitive and resistant strains were assayed in graded concentrations of the corresponding inhibitor, and dose-response curves plotted from cell counts at 7 days. Resistant lines showed an increase in maximal levels of analogue tolerated without reduction in cell number, as well as by a rise in drug concentration required for 50 percent growth inhibition at 7 days. Drug resistance was specific for the analogue used to treat the parent strain, and cross-reactions to the other two inhibitors used were minimal or lacking.—J. Nat. Cancer Inst. 26: 13–18, 1961.

THE EVOLUTION of simplified media (*1–3*) and methods for clonal analysis (*4, 5*) have opened a broad approach to the study of variation in isolated cell populations. Results already available indicate that nutritional variants (*6, 7*) may be obtained by this means, as well as populations resistant to antimetabolites (*8–12*). Drug resistance in particular offers a useful tool for continuing studies. Data obtained recently (*8, 10*) show that resistance in cell cultures, as *in vivo* (*13*), arises spontaneously, with the drug acting as a selective agent for variant cells. Such variants thus have the properties of mutants, though until a suitable means for analysis by recombination can be devised for somatic cells, the exact mechanism of variation remains obscure.

The use of plating procedures, as in microbiology, has made possible estimates of mutation rates for drug resistance in mammalian cells (*9, 11*) and preliminary characterization of the steps involved. These methods, however, are most useful for strains which grow in the form of compact, well-isolated colonies, and are less applicable to cell types with other growth forms. It is also clear that the incidence of resistant colonies in cultures treated with antimetabolites is affected by a number of variables, including the concentration of serum, age of assay cultures, persistence

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[2] This investigation was supported by grant RG-6025, National Institutes of Health, Public Health Service.
[3] With the technical assistance of Mrs. Irene Cady.

of sensitive or partially resistant cells, and size of colonies selected for scoring (*14*).

In view of these limitations in plating procedures, additional criteria are needed for the estimation of drug resistance in cell cultures. The present paper outlines the use of differential toxicity tests for this purpose. Data obtained on three cell strains indicate that the degree and specificity of resistance can be usefully expressed in index form.

MATERIALS AND METHODS

Culture techniques.—All experiments were performed with a strain of pig kidney cells first established by Dr. Edith Stice at Cutter Laboratories, Berkeley, California, in 1956. Two clones (PK 15, PK 33) derived from this line were used in the present investigation. Cultures of these cells were maintained in prescription bottles as static monolayers, with a nutrient of 10 percent lamb serum, 50 percent medium 199 (*1*) and 40 percent Earle's balanced saline solution. Further details on handling of cultures are included in a preceding publication (*15*).

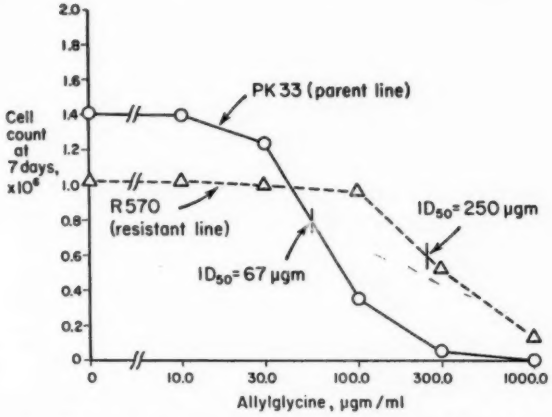
Derivation of resistant lines.—Three inhibitors were employed: 2,6-diaminopurine (DAP), aminopterin (AI), and allylglycine (ALG, *16*). Cultures were initiated at 1.0×10^6 cells, with one of these analogues present in the medium at a level which resulted in nearly complete destruction after 7 days of culture. For DAP this concentration was 100 μg/ml; for AI, 0.1 μg/ml; for ALG, 1 mg/ml. After exposure for a week, the inhibitor was removed, the surviving cells regrown to mass populations, and the process repeated. After three cycles of exposure, each strain could be propagated serially in the presence of the respective inhibitor, at the level used to treat the original sensitive population.

Assay procedures.—For estimation of resistance, subcultures from resistant lines were made into medium without inhibitor. From the resulting mass populations, assay cultures were set up with graded levels of the corresponding antimetabolites in 10 percent lamb serum and 50 percent medium 199. Two or three cultures were established at each concentration, with the use of 0.2×10^6 cells in 1-ounce prescription bottles. Parallel cultures were set up with cells from the original sensitive parental line (PK 15 or PK 33). After incubation at 37° C for 7 days with three fluid changes, the cultures were trypsinized and enumerated with a Coulter electronic cell counter (*15*). The resulting data were plotted in the form of log dose-response curves. The level of antimetabolite required for 50 percent growth inhibition (ID50) provided a convenient index of resistance. This could be read directly from dose-response curves by the calculation of the corresponding cell count, as follows:

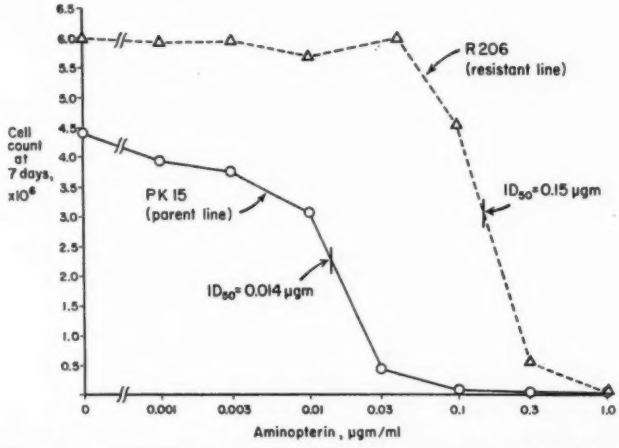
$$\text{Cell count (ID50)} = \frac{\left(\frac{\text{cell count at 7 days}}{\text{without inhibitor}}\right) - \left(\frac{\text{initial}}{\text{cell count}}\right)}{2} + \left(\frac{\text{initial}}{\text{cell count}}\right)$$

RESULTS

Resistance was observed with all three treated lines when differential toxicity tests were carried out with the corresponding inhibitors. Text-figures 1 and 2 show typical results obtained. Drug resistance was evidenced by an increase in the maximal level of analogue tolerated without growth inhibition at 7 days, as well as by a significant rise in drug concentration required for 50 percent growth inhibition (ID50). In practice, ID50 values were more easily and reliably determined than threshold levels for minimal inhibition. Cell lines resistant for DAP and ALG grew more slowly than the parental strain of origin (PK 15). No simple



TEXT-FIGURE 1.—Dose-response assays with sensitive and resistant lines of pig kidney cells. Line R570 derived by cyclic exposure to allylglycine as described in text.



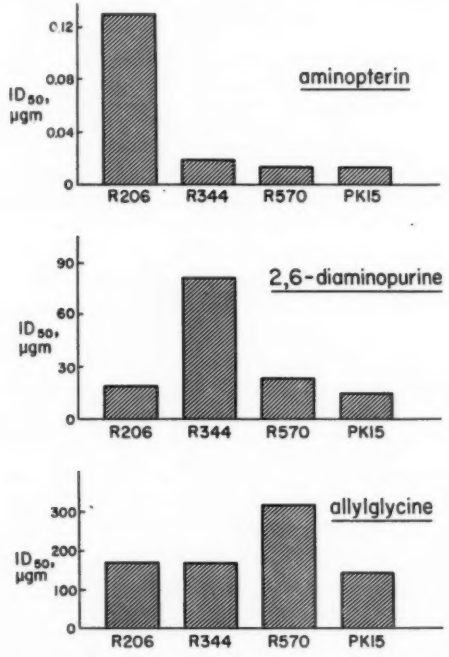
TEXT-FIGURE 2.—Dose-response assays with cell lines sensitive and resistant to aminopterin.

correlation existed between resistance and growth rate, however, since the strain resistant to AI proliferated more extensively than the corresponding sensitive line (text-fig. 2).

Table 1 provides a summary of representative data obtained in successive experiments with these cell strains. The ratio of ID50 values obtained on resistant and sensitive lines affords a convenient index for the estimation of the degree of resistance to a given inhibitor. Higher ratios were obtained for AI than for DAP or ALG under the conditions of this experiment.

Additional assays were carried out to determine the specificity of resistance to the three inhibitors used. ID50 values were determined for all three analogues on each resistant cell line. The results of this work are shown in text-figure 3. It is apparent that the responses obtained are selective for the inhibitor used to treat the parent strain initially. For ALG the differential response of the resistant line (R570) appears to be less than for AI or DAP. However, R570 is derived from PK 33 which is more sensitive than PK 15 and its derivatives to ALG (table 1, text-fig. 1).

These observations thus indicate that drug resistance is a useful physiological marker for the characterization of variant cell strains. This resistance persists for long periods in the absence of inhibitor and can be



TEXT-FIGURE 3.—Specificity of resistance to antimetabolites. PK 15: untreated by previous exposure to inhibitors. Other cell lines derived by cyclic treatment with allyglycine (R570), 2,6-diaminopurine (R344), and aminopterin (R206).

TABLE 1.—Drug resistance in populations of pig kidney cells treated with antimetabolites

Inhibitor	ID50 determinations*		ID50 ratio, resistant/sensitive
	Parental cell line	Treated† strain	
2,6-Diaminopurine	9	60	6. 7
	12	50	4. 2
	25	130	5. 2
	13	84	6. 5
Allylglycine	67	250	3. 7
	63	380	6. 0
Aminopterin	0. 012	0. 11	9. 2
	0. 014	0. 15	10. 7

*Concentration of inhibitor required for 50 percent growth inhibition at 7 days, μg/ml.
†After three cycles of exposure to inhibitor as specified in text.

demonstrated in clonal isolates (*14*). The assay system described provides a means of comparing the responses of sensitive and resistant lines to a given compound and an experimental design for further studies on cell variation.

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Method for Cytological Detection of Tumor Cells in Mouse Blood *In Vitro*[1]

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SUMMARY

A procedure for isolation of tumor cells from whole blood of mice is described, which utilizes Dextran and the Millipore filter technique. The cytological characteristics of isolated tumor cells are preserved and there is minimal loss of cells. Leukocytes and tumor cells can easily be differentiated and screened.—J. Nat. Cancer Inst. 26: 19–23, 1961.

THE ISOLATION of tumor cells from the blood of man has been accomplished by several methods (*1–5*) which have been satisfactorily applied in clinical studies. Methods for the isolation of tumor cells from the blood of animals must satisfy prerequisites similar to the methods used in human studies. Tumor cells must be isolated without destruction or morphologic alteration and collected on a small surface area to facilitate the task of screening. Because of the large number of specimens processed in precise experimental work, the methods employed in clinical studies were found impractical. While streptolysin-O lysed human erythrocytes and polymorphonuclear leukocytes (*2, 6*), it did not lyse erythrocytes and granulocytes of mouse blood. Saponin, as used by Engell (*1*), adequately lysed mouse erythrocytes, but mouse blood thus treated was difficult to filter through a Millipore membrane. Lysis of mouse erythrocytes with 2 percent acetic acid or distilled water resulted in more morphologic damage to tumor cells and leukocytes than in the method herein described.

The use of macromolecules for separation of leukocytes from blood has been studied extensively by Skoog and Beck (*7*). The separation depends on differences in sedimentation between leukocytes, platelets, and erythrocytes, due to differences in their specific gravities. The specific gravity of erythrocytes ranges from 1.092 to 1.097 (*5*) and is greater than that of leukocytes and tumor cells (*8*). Leukocytes have been shown to have a specific gravity varying from 1.07 to 1.08 (*9*), and human cancer cells about 1.056 (*5*). Maximum sedimentation rates of erythrocytes were

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obtained with a 6 percent solution of Dextran of molecular weight ranging between 139,000 and 228,000. Sedimentation in a 2:1 Dextran blood mixture resulted in yields of 76 ± 16 percent of leukocytes from whole blood. This method has been successfully applied by Chaplin *et al.* (*10*) to remove leukocytes from human blood. Separations of 83.9 percent of leukocytes by the first sedimentation procedure and 97.5 percent by 2 sedimentation procedures were achieved. This principle has been successfully applied in conjunction with the Millipore filter technique (*11*) for the isolation of white blood cells and tumor cells from the whole blood of 3 strains of inbred mice.

This article will report a method for the cytological detection of tumor cells in mouse blood, which utilizes Dextran and the Millipore filter technique. To test the quantitative aspect of the method, known quantities of two different transplantable tumors were added *in vitro* to mouse blood. The method has been successfully applied to studies *in vivo* of blood of mice bearing tumors (*12*).

PROCEDURE

Heparinized mouse blood from C57BL/6 non-tumor-bearing animals was used in preliminary studies. A cytosieved suspension of transplantable Sarcoma T-241, as carried in the C57 inbred strain of mice, was used as a test preparation. As determined by counts in a hemocytometer, 450 tumor cells were added to 1 cc whole blood collected from the right atrium of adult mice. One cc of 0.85 normal saline and 1 cc of 6 percent Dextran (mol. wt. 188,000)[4] were added to this mixture in a 50 ml conical centrifuge tube. The resultant mixture was mixed thoroughly and then allowed to settle for 40 minutes for sedimentation of erythrocytes. The supernatant (containing leukocytes and tumor cells) was removed as completely as possible. Saline, 0.85 percent, was added to the remaining red blood cells to restore the original 1 cc volume, and 1 cc volume of Dextran and 0.85 percent saline were added again. A second sedimentation was performed for 30 minutes. The combined supernatants were centrifuged at 1500 rpm for 7 minutes in a conical centrifuge tube and the fluid portion discarded. We resuspended the sediment (containing leukocytes and tumor cells) in 30 cc of 0.85 percent saline by gently taking it up into and expressing it from a 10 cc syringe through a 27-gauge needle. Five cc of 2 percent acetic acid was added to the suspension to lyse any remaining erythrocytes. After centrifugation at 1500 rpm for 7 minutes, the supernatant was removed, discarded, and the sediment plug again resuspended in 30 cc of 0.85 percent saline for further washing and removal of debris. Centrifugation and suspension were repeated twice. Ten cc of 10 percent formalin and 10 cc of 0.85 saline solution were added to the plug, and the resultant suspension was filtered at 20 mm Hg pressure through a Millipore filter, pore size 5 μ. The filters were immediately fixed in Carnoy's

[4] Dextran supplied by Pharmachem Corp., Bethlehem, Penna.

solution[5] for at least 10 minutes and stained according to the Papanicolaou technique, mounted in Permount on 52 × 77 mm slides, and scanned microscopically for tumor cells.

RESULTS AND DISCUSSION

Five 1 cc samples of mouse blood from C57 mice were mixed *in vitro* with 450 Sarcoma T-241 tumor cells, as determined by cell counts in a counting chamber, and were processed as described. The number of tumor cells recovered per preparation was 445, 481, 452, 417, and 430. Inherent in the pipetting and hemocytometer counting is a 5 to 10 percent error. Therefore, variations from 450 of ±10 percent may be considered equivalent to total recovery of tumor cells. The variation in this experiment was less than ±8 percent. To determine whether tumor cells were lost in processing, the red blood cells remaining after the Dextran treatment were hemolyzed with 2 percent acetic acid, washed, and filtered through separate Millipore filters, together with washings and supernatant fluids destined for discard. No tumor cells were found on examination of the filters.

This method was also tested with Sarcoma 49 in DBA mice. Five hundred and sixty-eight tumor cells in a blood preparation *in vitro* were processed, and 501, 555, 528, 564, and 536 tumor cells were recovered. One similar specimen of tumor inoculum not mixed with blood was promptly filtered to serve as a control, and 512 tumor cells were recovered. These values are within the ±10 percent range. Finally, 402 tumor cells of a cellular suspension of Cloudman S-91 melanoma were processed and 440, 362, 421, 382, and 403 tumor cells recovered on the individual Millipore filters. Variations within ±10 percent error were obtained.

The combination of Dextran for differential sedimentation, acetic acid for lysis of remaining erythrocytes, and utilization of the Millipore filter technique appear to fulfill the requirements of a procedure for the preparation of small samples of blood for detection of circulating tumor cells in small samples of mouse blood. Tumor cells, as well as white blood cells, were recovered in satisfactory morphologic condition (figs. 1, 2, and 3). The method is simple, inexpensive, and relatively rapid.

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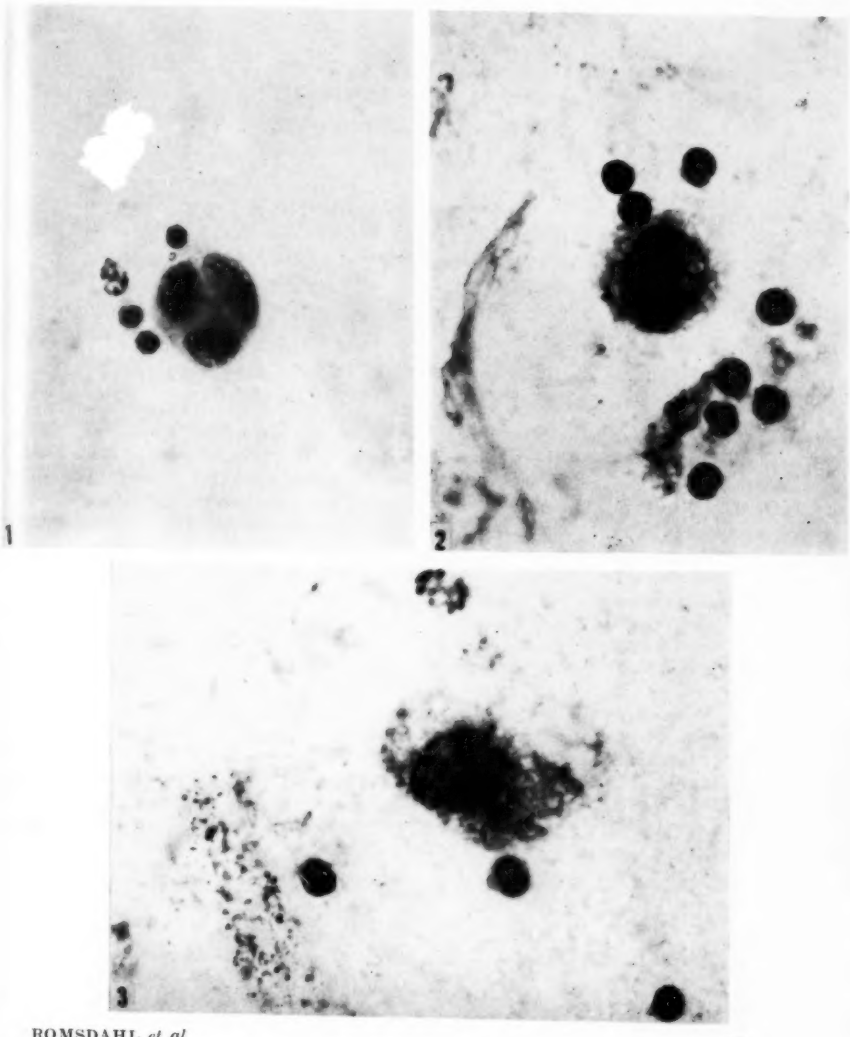
Plate 2

Figure 1.—Sarcoma T-241 tumor cells on Millipore filter after treatment with 6 percent Dextran.

Figure 2.—Single Sarcoma DBA-49 tumor cells on Millipore filter with smaller white blood cells.

Figure 3.—Tumor cells from Cloudman S-91 melanoma on Millipore filter after isolation with Dextran method.



Effect of Ehrlich Ascites Tumor on Mammary Glands of Mice of Low and High Mammary-Cancer Strains [1,2]

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SUMMARY

Ehrlich ascites tumor (EAT) transplants stimulated adjacent mammary-duct epithelium in C57BL mice. The presence of the ovaries and the consequent cyclic activity in the mammary gland during the course of the experiment did not modify the response of the mammary glands to the tumor stimulus. The stimulation consisted of cell enlargement and cell proliferation, with maximal mitotic activity on the 2d day after tumor inoculation. Inward proliferation of mammary-duct cells resulted in an increased height of the mammary-duct lining and diminution of duct lumen. Mammary-duct stimulation was confined to a 1 mm zone around the tumor transplant, but not all ducts in this area were affected. Greatest mitotic activity was found in mammary ducts which were not separated from the tumor transplant by an intervening layer of connective tissue. No apparent correlation existed between the genetic susceptibility of mammary glands to carcinogenesis and their sensitivity to the tumor stimulus. Mammary-duct cells of C3H mice, with a high incidence of spontaneous mammary cancer, were less responsive to the mitogenic effect of the tumor transplants than the mammary glands of the low mammary-cancer strain of C57BL mice. Mammary glands of DBA mice, another high mammary-cancer strain, resembled those of the C57BL strain.—J. Nat. Cancer Inst. 26: 25–35, 1961.

PREVIOUS REPORTS (*1*, *2*) from our laboratory showed that subcutaneous Ehrlich ascites tumor (EAT) transplants stimulated adjacent mammary-duct epithelium in adult ovariectomized C57BL mice. The inward proliferation of mammary-duct epithelium and cell enlargement resulted in an increased height of the mammary-duct lining. Maximum mitotic activity occurred on the 2d day after tumor inoculation, and mammary ducts were stimulated up to a distance of 1 mm away from the tumor nodule.

In the present experiments, we investigated whether the presence of the ovaries would modify the response of the mammary gland to the

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tumor stimulus. The mammary gland has been shown to undergo cyclic activity, in terms of cell division, as a result of hormonal changes during the estrous cycle (*3*). Our experiments would therefore test whether a mammary gland, which is undergoing cyclic activity during the course of tumor growth, responds differently to the tumor stimulus.

Because the mammary ducts, stimulated by adjacent tumor transplants, resembled descriptions by others of precancerous lesions (*4*), we decided to determine the effect of tumor transplants on the mammary glands of mice with a high incidence of spontaneous mammary tumors. It was felt that mice susceptible to mammary cancer might show a greater response to the tumor stimulus than the C57BL mice, which have a low incidence of mammary tumors.

MATERIALS AND METHODS

Three-month-old female C57BL, DBA/1, and C3H mice were used. The former 2 strains were bred, by brother × sister matings, in our own laboratory, while the latter mice were purchased from the Roscoe B. Jackson laboratory, Bar Harbor, Maine. Conditions for maintenance of the mouse colony and diet have been described in a previous publication (*2*). The EAT was similar to the one used in our earlier experiments (*1*, *2*).

The DBA/1 and C3H mice were ovariectomized 10 days before tumor inoculation. Daily vaginal smears were taken to ensure complete ovariectomy. Intact C57BL mice, with normal estrous cycles, were used. All mice received injections, under aseptic conditions, of 0.1 ml of tumor material, containing 15–30 × 10^6 cells, underneath the second left nipple. The technique of tumor implantation was described before (*2*). The contralateral mammary gland was inoculated with an equivalent amount of sterile saline and served as control.

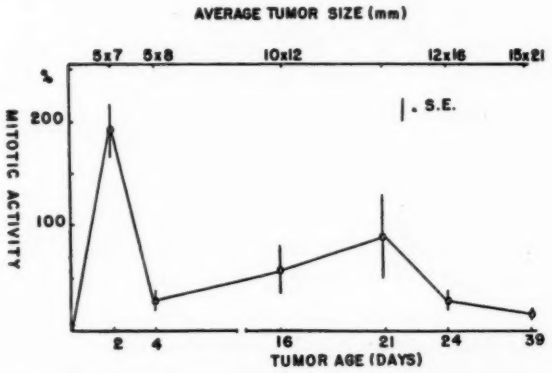
All mice were treated with a dorsal subcutaneous injection of 0.1 mg of colchicine (Fisher), freshly dissolved in 0.25 ml of saline, 6 hours prior to death. Animals were killed by cervical dislocation, vaginal smears were taken, and tumors were measured with calipers. Biopsy specimens were handled as before (*2*). Microscopic measurements were limited to mammary ducts located within a 1 mm zone around the tumor transplant. Mitotic activity was determined as the number of mitotic figures per total number of mammary ducts observed and expressed as percentage values. Measurements of mammary-duct diameter, height of mammary-duct lining, and distance from the tumor were made with an ocular micrometer.

RESULTS

Effect of Subcutaneous Ehrlich Ascites Tumor Transplants on Mammary Gland of Intact C57BL Mice

Results in this experiment indicated that the mammary-duct epithelium of intact mice responded in the same fashion to the tumor stimulus

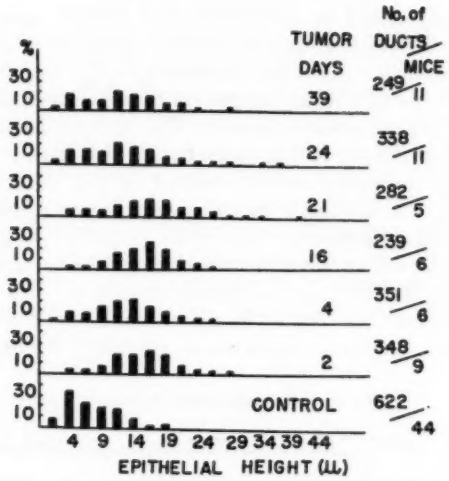
as the mammary gland of ovariectomized C57BL mice (*1*, *2*). Text-figure 1 shows the mitotic activity in the mammary-duct cells adjacent to tumor transplants. The mitotic activity (%) was calculated on the basis of the number of mitotic figures per total number of ducts observed, stimulated as well as nonstimulated. The mitotic activity was plotted against tumor age (days) and average tumor size (mm). The number of mice used and mammary ducts counted in the determination of the mitotic activity was the same as indicated in text-figure 2. There was a sharp rise in the mitotic activity (190 ± 26%) on the 2d day after tumor inoculation, with a subsequent decline until a level of 12 ± 5 percent was reached on the 39th day of tumor residence. An average mitotic activity of 6 percent was found in the saline-injected control mammary glands of all mice used during the entire course of the experiment.



TEXT-FIGURE 1.—Mitotic activity in mammary-duct epithelium of intact C57BL mice at various intervals after tumor inoculation. Mitotic activity expressed as number of mitotic figures per 100 mammary ducts. Average tumor size refers to the average of tumor diameters.

Text-figure 2 illustrates the effect of tumor residence on the degree of mammary-duct stimulation, expressed in terms of epithelial height. After 2 days of tumor residence there was an increase in the number of stimulated ducts. This stimulation persisted for approximately 3 weeks and then began to decline. The distribution chart also indicates that not all ducts in or around the tumor nodule became affected.

Histologically, the tumor nodule consisted of a central area of necrosis, a peripheral rim of actively dividing tumor cells, and a surrounding layer of metachromatic connective tissue (fig. 1). Necrosis and connective-tissue capsule were already present on the 2d day after tumor inoculation (fig. 2), but whenever the tumor cells were infiltrating fatty tissue, no connective-tissue capsule formed (fig. 3). The mammary ducts located in these areas were therefore not separated from the tumor by a connective-tissue capsule, and it was in such ducts that the highest mitotic activity was found (fig. 4). At later stages of tumor growth a connective-tissue



TEXT-FIGURE 2.—Range and frequency distribution (%) of epithelial heights in mammary ducts at various intervals after tumor inoculation.

capsule was usually present. At this time the mitotic activity in the mammary-duct epithelium had decreased (text-fig. 1), but many of the mammary ducts still had their stimulated appearance (fig. 5). Figure 6 represents a control mammary duct in the contralateral gland.

Effect of Subcutaneous Ehrlich Ascites Tumor Transplants on Mammary Glands of DBA/1 and C3H Mice

Both the DBA/1 and C3H mice are susceptible to spontaneous mammary-cancer formation (*5*). Table 1 records the effects of subcutaneous EAT transplants on the mammary-duct epithelium of these mice susceptible to mammary cancer. The mammary-duct stimulation, in terms of mitotic activity, in the DBA/1 mice was quite marked (fig. 7) and not significantly different from that in the C57BL strain. The mitotic activity rose to 239 percent on the 2d day of tumor growth, and declined to a value of 3 percent on the 24th day after tumor transplantation. As in the C57BL mice, the increased mitotic activity resulted in an increased height of mammary-duct lining (fig. 8). Twenty-four days after tumor implantation, the range of epithelial heights had increased to an upper limit of 26.4 μ. In spite of this increased *range* in epithelial height, at 24 days, the *average* epithelial height showed a decreased value. This was due to the reversing trend in mammary-duct stimulation, which, as noted before, becomes apparent after approximately 3 weeks of tumor residence.

The C3H mice, at 2 days, were less responsive in terms of mitotic activity to the tumor stimulus than the DBA/1 mice, even though the susceptibility of intact mice of this strain to mammary carcinogenesis is greater (*5*). The mitotic activity in the mammary-duct epithelium of these mice rose

TABLE 1.—Effect of subcutaneous Ehrlich ascites tumor transplants on adjacent mammary-duct epithelium of 10-day ovariectomized DBA/1 and C3H mice

Mouse strain	Days of tumor growth	Number of mice	Average tumor size (*mm*)*	Mammary ducts					
				Number	Lining				
					Number of layers	Height (μ)		Mitotic activity (% ± standard error)	
						Range	Average ± standard error		
DBA/1	2	14	4.5 × 7	472	1–3	2.4–21.6	10 ± 0.2	239 ± 46	
	24	6	12 × 15	189	1–4	2.4–26.4	9.3 ± 0.3	3 ± 1	
	Control	27	—	353	1–2	2.4–12	6.2 ± 0.1	0	
C3H	2	12	4.5 × 8	351	1–3	2.4–16.8	9.6 ± 0.2	77 ± 25	P†–0.006
	24	9	16 × 22	364	1–3	2.4–21.6	8.9 ± 0.2	8 ± 2	
	Control	23	—	306	1–2	2.4–14.4	5.6 ± 0.1	0	

*Average tumor size refers to the average of tumor diameters.
†P value determined by the small sample method of Fisher.

to only 77 percent on the 2d day after tumor inoculation (table 1) and then declined to reach a level of 8 percent on the 24th day of tumor residence. Even though the mitotic activity in the C3H mammary gland at 2 days was considerably less than in the DBA/1 mice, an increase in the height of mammary-duct lining was still apparent, as is shown in the lower portion of table 1. A comparison of the range of epithelial heights between the 2 strains indicates a somewhat greater degree of mammary-duct stimulation in the DBA/1 mice. When one compares the averages of epithelial heights, however, this difference in degree of mammary-duct stimulation is obscured.

Tumor measurements indicated that at 2 days the rate of tumor growth in the DBA/1 and C3H mice was comparable (table 1), but at 24 days, the tumors in the C3H mice were somewhat larger. Histologically, the transplants in the 2 strains were quite similar throughout the experiment. Necrosis and connective-tissue capsule formation were noticeable by the 2d day after tumor inoculation except where the tumor was infiltrating fat tissue. The mitotic activity in the mammary-duct epithelium was highest in the absence of an intervening connective-tissue layer between tumor and mammary ducts. Mammary-duct stimulation was again confined to an approximate zone of 1 mm around the tumor nodule, but not all ducts in this area were affected.

DISCUSSION

These studies stemmed from earlier observations that subcutaneous transplants of Ehrlich ascites tumor (EAT) stimulated the growth of adjacent mammary tissue in ovariectomized C57BL mice (*1, 2*). The results indicated that the presence of the ovaries, with their hormonal stimuli, did not modify the response of the mammary gland to this tumor stimulus. The mitotic activity in the mammary-duct epithelium of intact tumor-bearing mice rose to a peak on the 2d day after tumor inoculation, rapidly declined to return to slightly above normal in about 3 to 4 weeks. Inward proliferation and cell enlargement of the mammary-duct cells resulted in intraluminal projections and/or partial obliteration of the duct lumen. Because these stimulated mammary ducts resembled the descriptions by others of early mammary carcinoma, the effect of subcutaneous tumor transplants on the mammary glands of high mammary-cancer strain of mice was studied. Our results indicated that there was no apparent correlation between the sensitivity of the mammary glands of 10-day ovariectomized C3H, DBA/1, and C57BL mice to the tumor stimulus and the respective incidence of spontaneous mammary carcinogenesis in intact mice of these strains. The mammary-duct epithelium of ovariectomized C3H mice was less responsive to the mitogenic effect of a 2-day-old tumor transplant than the mammary tissue of ovariectomized DBA/1 and C57BL mice. Both the C3H and DBA/1 mice have a high incidence of spontaneous mammary-cancer formation, whereas the C57BL mice belong to a low mammary-cancer line.

The mammary-duct stimulation in all 3 strains occurred up to a distance of 1 mm away from the tumor nodule. This suggested that a diffusible agent might be involved. Usually the tumor transplant consisted of a central area of necrosis, a peripheral rim of viable tumor cells, and a surrounding layer of connective tissue. Whenever the tumor nodule was infiltrating fatty tissue, however, the connective-tissue capsule was absent. Mammary ducts located in this area were therefore not shielded from the tumor cells by an intervening layer of connective tissue. These mammary ducts appeared to exhibit a much higher mitotic activity than the ducts which were separated from the tumor by a connective-tissue barrier. At the later stages of tumor growth, a connective-tissue capsule was almost always present.

The decreased mitotic activity in the mammary-duct epithelium during the later stages of tumor growth might well be due to the formation of a heavier connective-tissue capsule, which prevented the escape of the stimulating factor(s). Malmgren (*6*), who demonstrated a mitogenic effect of subcutaneous tumor transplants on the liver, found a similar decline in mitotic activity when the tumor exceeded a certain size. But if this same tumor was homogenized and reinjected, mitotic stimulation of the liver cells took place once more. These results suggest that the growth-promoting activity of the older tumor was not lost but was perhaps confined by the surrounding connective-tissue capsule.

The different sensitivity to the tumor stimulus of C3H mammary tissue as compared to that of C57BL and DBA/1 mice might be due to a differential sensitivity of the target organ. Experimental evidence for such a differential sensitivity of the target organ has been provided by others to explain strain differences in vaginal sensitivity to estrogen (*7*), in estrogen-induced tumor formation of the testis (*8*), and in spontaneous mammary-cancer formation (*9*). The different response of the C3H mammary gland, however, could also be due to a variation in tumor growth rate and/or pattern. Studies on the mechanism of the mammary-duct stimulation by tumor transplants, now submitted for publication, suggest that living, but not necessarily dividing, tumor cells are required for the mammary-duct stimulation and that ascitic fluid, necrosis, or connective tissue by itself is not responsible.

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PLATE 3

FIGURE 1.—Histology of 16-day-old subcutaneous Ehrlich ascites tumor transplant. *Note* central area of necrosis, peripheral rim of growing tumor cells, and surrounding connective-tissue capsule. Toluidine blue. × 50

FIGURE 2.—Histology of 2-day-old tumor transplant illustrating central necrosis on *right,* and surrounding connective-tissue capsule on *left* of viable tumor cells. Toluidine blue. × 500

FIGURE 3.—Two-day-old tumor transplant infiltrating fatty tissue. *Note* absence of connective-tissue capsule. Toluidine blue. × 30

FIGURE 4.—High mitotic activity in C57BL mammary duct located next to 2-day-old tumor transplant of type described in figure 3. In absence of intervening connective-tissue layer, mitotic activity in mammary-duct epithelium was highest. Toluidine blue. × 150

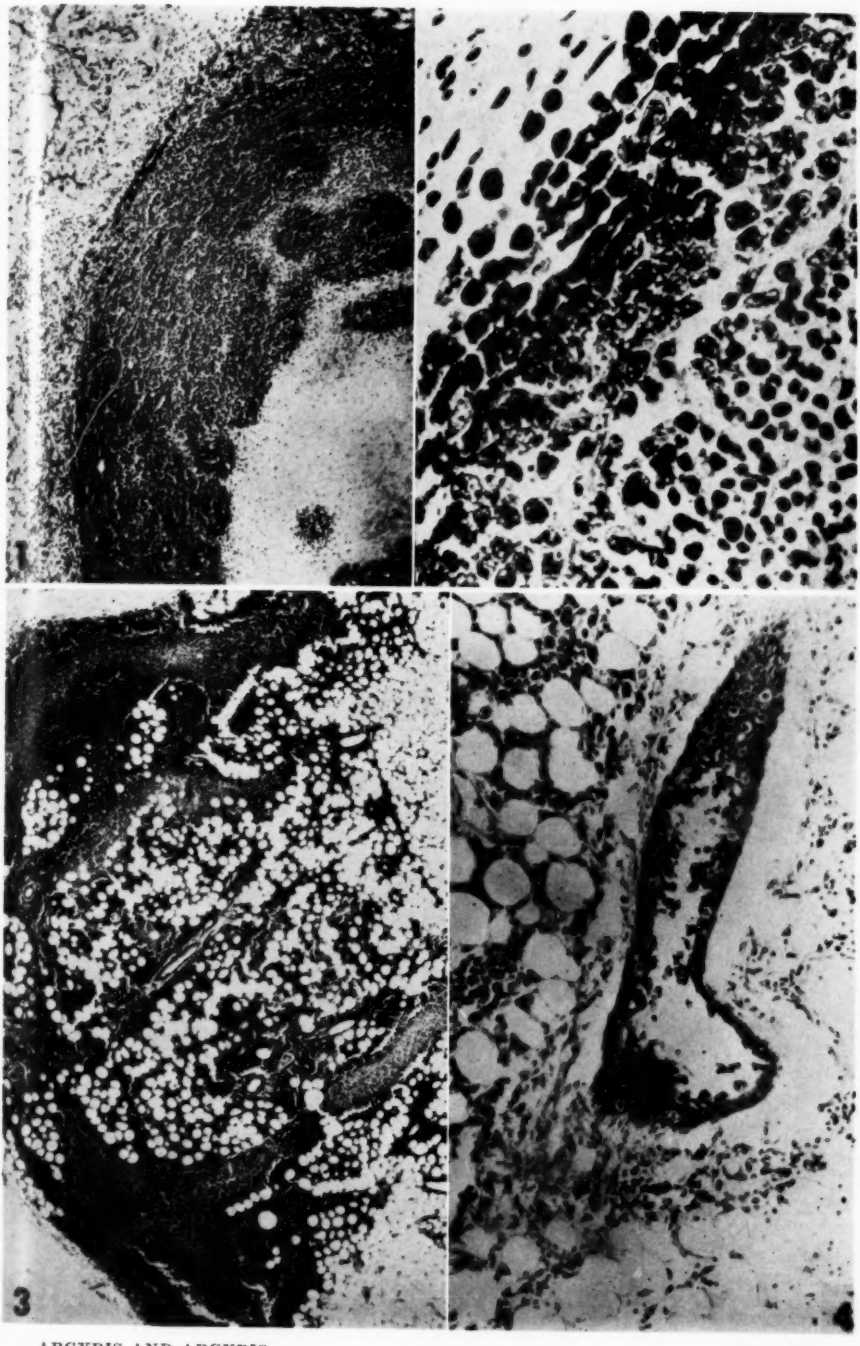
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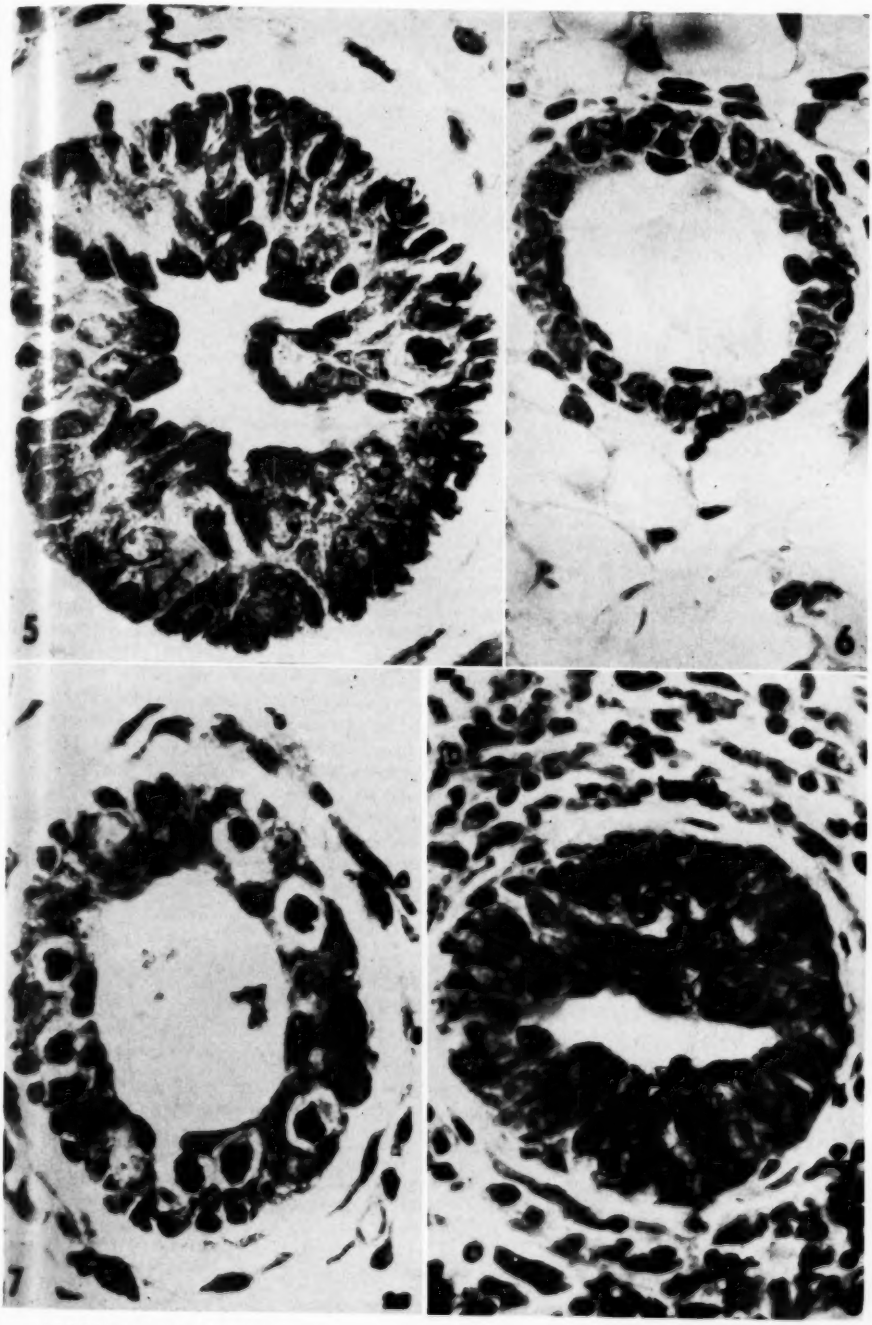
PLATE 4

FIGURE 5.—Stimulated C57BL mammary duct located 200 μ away from 16-day-old EAT transplant. Lining in this 70 μ-wide mammary duct measured 24 μ. *Arrows* indicate colchicine-arrested mitotic figures. *Note* cell enlargement and intraluminal projections. Toluidine blue. × 1,150

FIGURE 6.—Normal control mammary duct in contralateral gland of tumor-bearing C57BL mouse. *Compare* height of epithelial lining and cell size with that in figure 5. Toluidine blue. × 1,000

FIGURE 7.—Mammary duct located 500 μ from 2-day-old EAT transplant in castrated DBA/1 mouse. Darkly stained cells represent colchicine-arrested mitotic figures. Toluidine blue. × 1,000

FIGURE 8.—Increased mitotic activity and cell enlargement resulted in increased height of epithelial lining and diminution of duct lumen. This mammary duct was 60 μ wide and was located inside a 24-day-old EAT transplant. Lining on *left* measured 26.4 μ and the one on *right* 33.6 μ. Toluidine blue. × 830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Cancer Mortality Among the Foreign-Born in the United States [1]

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SUMMARY

More than 34,000 cancer deaths recorded for foreign-born whites in 35 States during 1950 were analyzed by country of birth of the decedent. Results for 16 sites have been presented in the form of age-adjusted mortality ratios for migrants from twelve countries, expressed as percentages of the rates for native whites born in the United States. The foreign-born were in general subject to different schedules of cancer risk than native whites. Excesses or deficits in risk were not uniformly distributed by site across all ethnic groups, but were specific for certain site and ethnic-group combinations. Several of the more striking ethnic group differences were concentrated in the upper digestive tract—esophagus and stomach—in contrast to smaller variability by ethnic group for intestines or rectum. These ethnic-group effects were not confined to any single region of the United States and most of them persisted when data for six large metropolitan areas were examined separately. The consistency of the present results with earlier American reports on ethnic group differences in cancer risk is discussed. Some of the data on migrants from foreign countries have been compared with the results of experience in the countries of origin. Some areas of agreement and disagreement between the ethnic-group results and information on international variation in cancer mortality rates, as well as other findings on the epidemiologic characteristics of individual sites are also indicated.—J. Nat. Cancer Inst. 26: 37–132, 1961.

THE MIGRATION of human populations may be looked upon as one of nature's unplanned experiments. Several investigators have recognized the importance of utilizing opportunities to compare the morbidity and mortality experience of natives and migrants residing in the same areas to elicit information on the potential role of environmental and endogenous factors in specific diseases. Steiner (*1*) has stated the advantages for cancer research as follows:

> It is impossible, of course, to perform planned experiments on the etiology of cancer in man. Members of certain races have, however, unwittingly

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[2] I am indebted to Mrs. Margaret Crittenden for assistance in supervising the analysis of the U.S. mortality data.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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> performed etiological experiments on a large scale by migration from one environment to another. The genetic characteristics of such a population may at first remain relatively unchanged, but the new environment may be different from the old in some respects. Factors such as climate, altitude, air pollution, humidity, temperature, amount of solar and other types of irradiation, and intercurrent disease may differ at once. These factors might affect man both directly and also indirectly through his skin, air, food and water supply, and possibly in other ways. On the other hand, certain environmental factors, some of which may be cultural, change more slowly after migration. The choice of food and culinary practice, occupational exposures, sanitary habits, economic level, and other factors may gradually change over a period of years . . . .

In view of the clear understanding of the theoretical implications, it is surprising how little analysis of mortality data by country of birth of the decedent has been attempted in the United States, the destination of one of the great population movements of modern times. Federal and State vital statistics offices have paid far more attention to country of birth in the compilation of birth and marriage statistics; the U.S. Bureau of the Census, for example, published tables for births specifying the country of birth for each parent from 1900 to 1936.

The annual reports of the Bureau of the Census and the National Office of Vital Statistics (*2*) tabulated total deaths among native-born and foreign-born whites from 1900 to 1941 and again in 1950; in the latter year information on deaths from cancer (all sites) and other causes was also presented. Few details on country of birth of the decedent, or decedent's parents, have ever been supplied. In fact, the item was coded and entered on the tabulation cards for deaths in selected States and cities in only 4 years—1900, 1910, 1920, and 1921. The 1910 data by country of birth of decedent for New York and Pennsylvania, including references to cancer mortality, were the subject of three papers by Dublin (*3–5*). The 1920 cancer death rates by age, sex, and country of birth of decedent's mother (*i.e.*, a combination of foreign-born and native-born of foreign parentage) for New York State, New York City, Pennsylvania, Philadelphia, and Chicago were published in "Mortality Rates, 1910–1920." Data on mortality in 1900, 1910, and 1920 from cancer and other causes among white persons in Boston, whose mothers were born in Ireland and the United States, were appended to the 1921 annual report.

State and local vital statistics offices contributed little additional information. The early work of Davis, reporting substantial differences in the mortality rates from cancer and other causes among the foreign-born groups in Boston as of 1900 (*6*), did not attract wide attention. The next series of tabulations incorporating detail on country of birth of white decedents for cancer and other causes appeared in a 1925 report of the New York State Department of Health (*7*); the Department also prepared corresponding unpublished tables of cancer deaths covering the years 1920 and 1926–32.

Although these early and fragmentary vital statistics did not deal with individual sites of cancer, some promising leads emerged. Dublin found virtually all the foreign-born groups had higher risks of cancer than the

native-born, Italians constituting the only exception. The excess rates among the Irish were exceptionally striking; the risks were also higher among persons born in England and Germany, but not to the same degree. Other sources yielded similar findings. The 1921 report of the Bureau of the Census noted that the higher rates of cancer mortality for northern States could be partially accounted for by the greater proportion of foreign-born in that region. Variation in accuracy and reliability of death certifications were discounted at the time as a likely explanation for the differentials in ethnic groups; the 1921 text on this point reads:

> What possible reason, for example, is there for believing that certification for those whose mothers were born in the United States was poorer than for those whose mothers were born in Ireland?

Lombard and Doering (*8*) were the first to provide a systematic coverage of sites in a review of cancer mortality among the foreign-born in Boston. Their data suggested that the pattern of ethnic-group relationships varied with site. These authors, after considering the nature of the diagnostic evidence underlying the certifications, also thought that diagnostic factors could be ruled out as a possible explanation. Their discussion and interpretation emphasized the association between nativity group and social class and the potential role of economic and social conditions in the cause of cancer.

Lombard and Doering's results were not followed up at the time. For the next 25 years studies of ethnic-group variation remained a neglected field, with the exception of some inquiries into cancer risks among Jews and occasional studies of diagnosed cases and controls which took nativity into account, such as the one on cervix by Smith (*9*).

In recent years renewed interest in examining death certificates to compare cancer mortality among native- and foreign-born groups has been manifested. Shortly after Eastcott's (*10*) publication in 1955 on cancer mortality by site among natives and immigrants in New Zealand, Mancuso and Coulter (*11*) assembled data in Ohio on cancer of the lung, larynx, bladder, and central nervous system. MacMahon and Koller (*12*), to study ethnic-group differences in leukemia incidence, surveyed death certificates and hospital records in New York City. MacMahon (*13*) later extended his investigations of the New York City mortality data to cover other sites. Buechley *et al.* (*14*) were stimulated by Steiner's (*1*) finding of an unusually low male-female ratio for lung cancer among Mexicans in a necropsy series to examine death certificates in California; for this purpose they used a 1950 census report on the population with Spanish surnames (*15*) and classified the lung-cancer deaths in a comparable manner. Ethnic-group differences were also a major concern of a recent survey of cancer incidence among French Canadians (*16*). A controlled retrospective study of admissions to Roswell Park Memorial Institute in Buffalo, New York, indicated an excess of persons born in Poland among those diagnosed with cancer of the stomach (*17*).

In addition to direct observations of ethnic groups, collateral evidence on inter-area variation in cancer risk has been assembled (*18–20*) which

suggests that, for certain sites, differences in the composition of ethnic groups could be a factor contributing to geographic variability.

The reports cited, despite the relatively small number of observations, as well as omissions in coverage of ethnic groups, have yielded provocative findings meriting further investigation. The development of hypotheses to account for the differences and the design of studies to test them would be facilitated if the facts could be ascertained with greater precision and detail. The present investigation based on 34,463 cancer deaths recorded among foreign-born whites in the United States during 1950 was undertaken with this objective in mind and is on a larger scale than any hitherto attempted.

MATERIAL AND METHODS

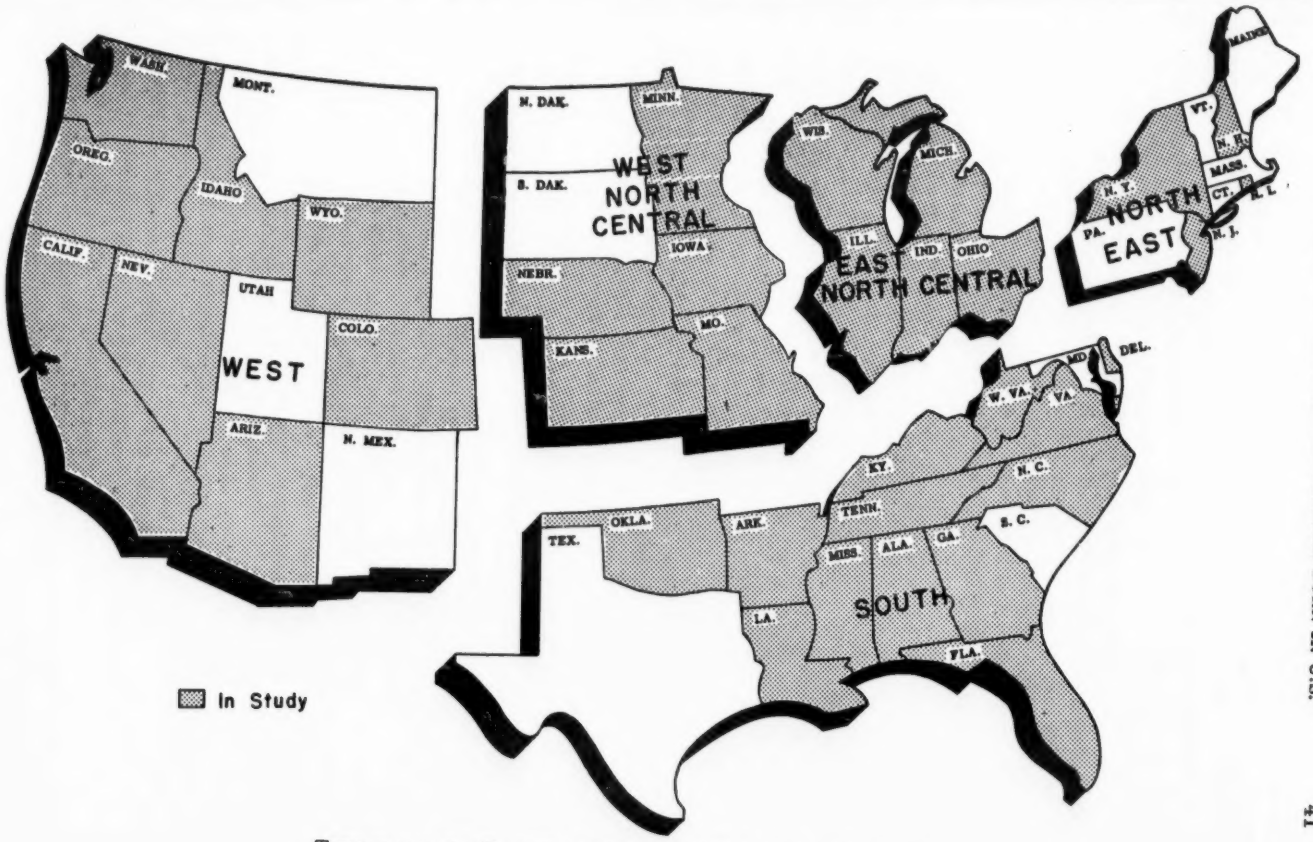
Death records.—The punch cards for all cancer deaths (International List #140–205, sixth revision) occurring in the United States during 1950 were obtained from the National Office of Vital Statistics, U.S. Public Health Service. The entries on race and general nativity were used to eliminate nonwhites and to distinguish between deaths of native- and foreign-born whites. For the 35 States indicated in text-figure 1, the death records were microfilmed for transmittal to the National Office of Vital Statistics, and the microfilm images for the foreign-born deaths were reviewed. The decedent's country of birth was coded according to the 1950 population census classification (*21*) and added to the punch card. For the remaining 13 States and the District of Columbia the punch cards were discarded, since the corresponding returns to the National Office in the form of handwritten transcripts were no longer available for reference.

The States included in this study accounted for 75 percent of all cancer deaths among foreign-born whites in the United States during 1950. All regions were represented, with coverage of the North Central States being particularly good, and the present material may be regarded as descriptive of that to be obtained from complete national data.

The scope of this inquiry could not be extended to consider differences in cancer mortality between the native-born of native parentage and of foreign or mixed parentage, since only seven States (California, Connecticut, Idaho, Louisiana, Rhode Island, Texas, and Utah) had retained birthplace of decedent's parents on the death certificate forms used in 1950.

No problem concerning consistency of cause-of-death coding arose in these contrasts of native- and foreign-born whites, since all the certificates had been coded in a uniform manner by the National Office of Vital Statistics.

Population estimates.—The age-sex distributions for the native- and foreign-born white from all countries were obtained from the 1950 census data published for each of the 35 States (*22*).



TEXT-FIGURE 1.—Regional distributions of States included in study.

While the 1950 census reports did indicate the total population by country of birth residing in each State, the age-sex distributions were tabulated only for 12 major foreign-born groups by geographic division and selected Standard Metropolitan Areas (*23*). Age-sex estimates were derived for each of these 12 groups by prorating the total counts for the constituent States in each division included in the study against the corresponding divisional age-sex distributions. The figures by division were then consolidated into totals for the 5 regions indicated in text-figure 1 and for the 35 States combined. The accuracy of the estimates for the limited number of States in the Northeast was improved by adjustment to regional population data, which excluded the Boston, Philadelphia, and Pittsburgh Standard Metropolitan Areas.

Standard Metropolitan Areas.—Since there are substantial urban-rural gradients in risk for certain cancer sites, the concentration of the foreign-born in urban centers made it desirable to investigate the effects associated with place of residence on contrasts between native- and foreign-born. Unfortunately, the Bureau of the Census, in reporting on the 1950 population according to country of birth, used its "new" definitions of urban and rural for which comparable mortality tabulations could not be produced. It was possible, however, to examine separately the experience for residents of 6 Standard Metropolitan Areas (Chicago, Cleveland, Detroit, Los Angeles, San Francisco-Oakland, and New York-northeastern New Jersey), and for persons residing outside these conurbations.

Mortality rates and ratios.—Age-, sex-, and site-specific mortality rates were computed for the native-white, total foreign-white, and the 12 major foreign-born groups in the 35-State area. While not all the age-specific rates for the latter groups appear in this paper, much of the material is presented in text-figures 7 to 10, which contrasts the U.S. experience of certain immigrant groups with the mortality prevailing in the countries of origin. The rates for foreign countries (also used to calculate the Standardized Mortality Ratios given in table 7) were taken from compilations published by Case and Harley (*24*) and Segi *et al.* (*25*).[4]

The variety of potential contrasts suggested the use of age-adjusted rates to summarize the detailed findings. Because of the concentration of the foreign-born in the older ages, a direct method of age adjustment—weighting the age-specific rates by a standard population—was rejected in favor of the indirect method of applying a standard schedule of rates to each population group to calculate an "expected" number of deaths. The observed deaths could then be divided by the corresponding "expected" numbers and the results expressed in the form of a Standardized Mortality Ratio (SMR).

Three sets of standard rates (U.S. native whites in each of 5 regions, total whites in 6 Standard Metropolitan Areas, foreign countries) leading to entirely different SMR's have been employed in this paper. The base for the SMR's has been indicated in the table and text-figure captions, which should be read carefully with this point in mind. Since recorded

[4] I am indebted to Dr. Segi for placing at my disposal unpublished age-specific rates for several sites.

cancer mortality varies by region within the United States, a prominent feature being higher rates for several sites in the Northeast, the SMR's measured in terms of native-white experience in table 3 and text-figures 4 to 6 were prepared by pooling separate regional determinations of "expected" deaths and thus have been effectively adjusted for regional distribution of the foreign-born as well as for age. The regional distribution of SMR's is presented in an appendix to this paper. The purpose of the appendix is not to give a direct measure of regional variation in risk for each foreign-born group (for this, use of a single set of standard rates would be required), but to emphasize the fact that the differentials between the migrant groups and the native-born populations for certain sites and ethnic groups are national characteristics and not confined to any one part of the United States.

Tests of significance.—The SMR's for foreign-born groups, which deviated significantly from the base rate of 100 for native whites at the 1 and 5 percent levels of significance for individual comparisons, have been so designated in table 3. Chance factors alone may produce some results apparently out of the ordinary from a large series of comparisons. In this fashion one might anticipate the 408 ethnic-group contrasts in table 3 to yield on the average 4 and 20, respectively, as meeting the 1 and 5 percent test criteria in the absence of true intergroup differences. In actual fact, 149 and 197 SMR's were found to be significant at these levels and collectively they would argue against attributing to random sampling variation all the divergent cancer risks noted among the foreign whites.

No tests of significance were applied to the SMR's based on mortality rates in foreign countries presented in table 7, since systematic biases arising from national differences in death registration and medical certification practices would be far more serious potential sources of error than sampling variation.

Consistency of death certificate and census information.—While the death certificates and census returns undoubtedly contained inaccuracies in statements of birthplace, under normal conditions such errors would assume importance only if there were marked, systematic discrepancies between the source documents. If, for example, there was a strong tendency to report persons from abroad as native-born in the population census, their mortality rates would be inflated above the true values. This conjecture of bias cannot be dismissed out-of-hand as unreasonable, since some might mistakenly believe that being recorded as native-born in the census could be advantageous.

To investigate the consistency of the nativity information, a sample of 1,500 cancer deaths occurring in June, 1950, immediately after the decennial census, was drawn. To reduce the labor of searching census records, the study was limited to 3 States, New York, Ohio, and Wisconsin, which sufficed to provide representation of most foreign-born groups. Since the primary concern was with effects on rates for specific ethnic groups, approximately 80 percent of the sample was alloted to decedents described as foreign-born. Seventy-eight percent of the deaths were successfully

TABLE 1.—Comparison of 1,173 death certificate statements on nativity with the 1950 population census returns for the same individuals

1950 Census	Death certificate			
	Born in foreign country		Born in United States	
	Number	Percent	Number	Percent
Total matched	963	100.0	210	100.0
Born in foreign country	926	96.1	5	2.4
a. The same country named on the death certificate	841	87.3	—	—
b. Not the same country named on the death certificate	85	8.8	—	—
Born in the United States	37	3.9	205	97.6

matched with census entries, a percentage typical for studies of this type carried out by the Bureau of the Census. Table 1 summarizes the findings.

The correspondence on nativity, *i.e.*, whether native- or foreign-born, was quite good. Only 42 census entries, less than 4 percent, contradicted the death record on this point; furthermore, both types of discrepancies appeared and compensated for each other, so that the net error rate would be still lower. Applying the sample results to the U.S. mortality data leads to the conclusion that adjustment for inconsistent reporting of nativity would have but little effect (on the order of $\pm 1\%$) on the cancer mortality rates for native whites. The impact on rates for total foreign whites could be somewhat greater, but allowance for this factor would not reduce them by much more than 3 percent.

For 87.3 percent of the foreign-born deaths the documents agreed on the specific country of birth. Most of the differences involved Poland, Austria, Germany, and neighboring states and tended to balance out in a reconciliation of the summary distributions of birthplace derived from the two sources. The greatest disparity noted was for Poland, which was mentioned 129 times on the death certificates versus 115 entries on the population schedules. Whether a systematic difference of this magnitude would be confirmed by a more extensive investigation is unknown, and too much weight should not be attached to this observation, which might easily be due to sampling variation. At worst, it suggests the possibility that the SMR's for Polish migrants might be overstated by 10 to 15 percent. Clearly, in the search for large differences in cancer risk among ethnic groups (on the order of $\pm 25\%$ or more) inconsistent reporting of data on census and death records may be ruled out as an important source of error.

RESULTS

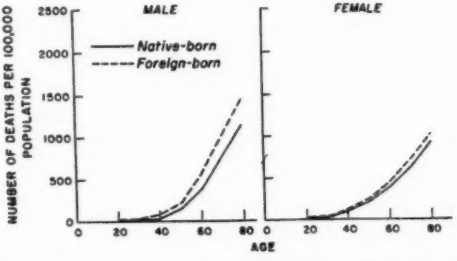
The primary concern of this paper is with patterns of variation in cancer risk by site among immigrants from 12 countries for whom more refined

comparisons in the form of age-adjusted mortality ratios could be made. Its scope has been limited to 16 sites and site combinations for which there were sufficient numbers of deaths to warrant examination of intergroup variability. The presentation of results has been accompanied by references to other pertinent mortality data reported by other investigators.

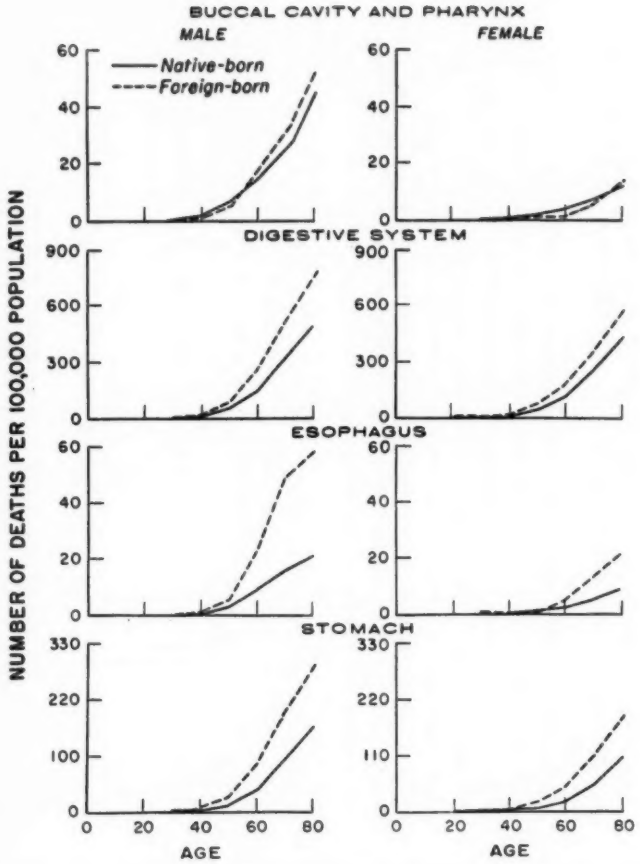
Native- and Foreign-Born Whites

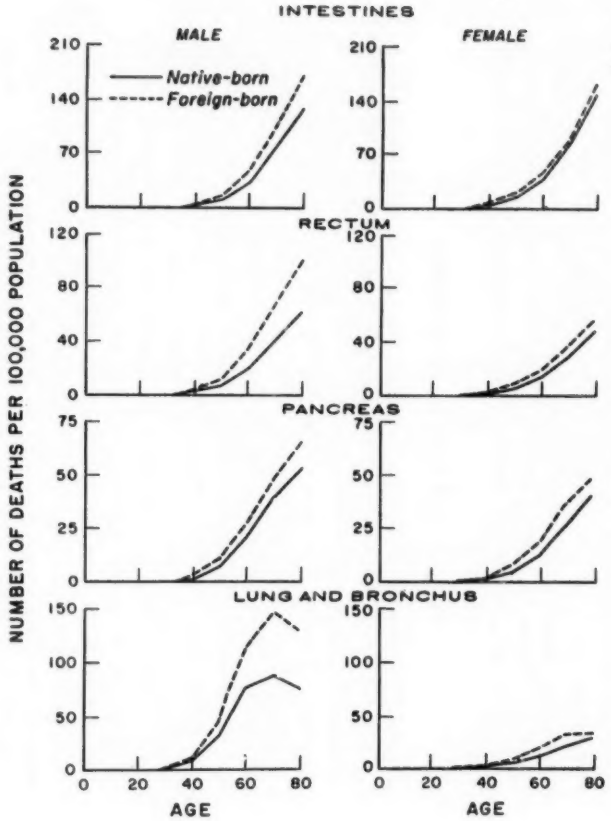
Before consideration of specific migrant groups, background information on some of the more general differentials in cancer mortality which characterize contrasts between the native- and foreign-white populations is supplied. Text-figures 2 and 3 present the age- and sex-specific mortality rates for all sites and selected sites, and the age detail is reduced to SMR's for total foreign-whites in the first column of table 3. For cancer, all sites, mortality was higher among the foreign-born, with the excess being more marked among males (24%) than among females (6%); these relationships tended to persist within the separate age groups as well. The present data confirmed previous findings of an over-all excess in cancer mortality among the foreign-born, particularly for males (table 2). The consistently higher foreign-born mortality for all sites, and for the majority of specific sites, would argue against interpreting all the ethnic-group excess risks as artifacts arising from practices for classifying foreign countries, since at least one constituent ethnic group must contribute to the over-all foreign-born result. The same line of reasoning would also apply to site distribution; excess risks for all sites combined must be accounted for by above-average experience in one or more sites.

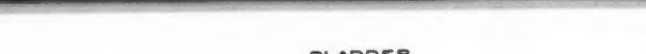
Three sources which classified the native-born by parentage *(6, 8, 26)*, a distinction not possible in 1950 for reasons stated, indicated a gradient in risk for all sites, from foreign-born to native-born of foreign parentage to native-born of native parentage. While the foreign-native differential in cancer mortality has apparently been diminishing during the past half century, particulary among males, the narrowing gap may be partly attributable to increased representation of persons of foreign parentage among the native-born. This conjecture is supported by the more recent

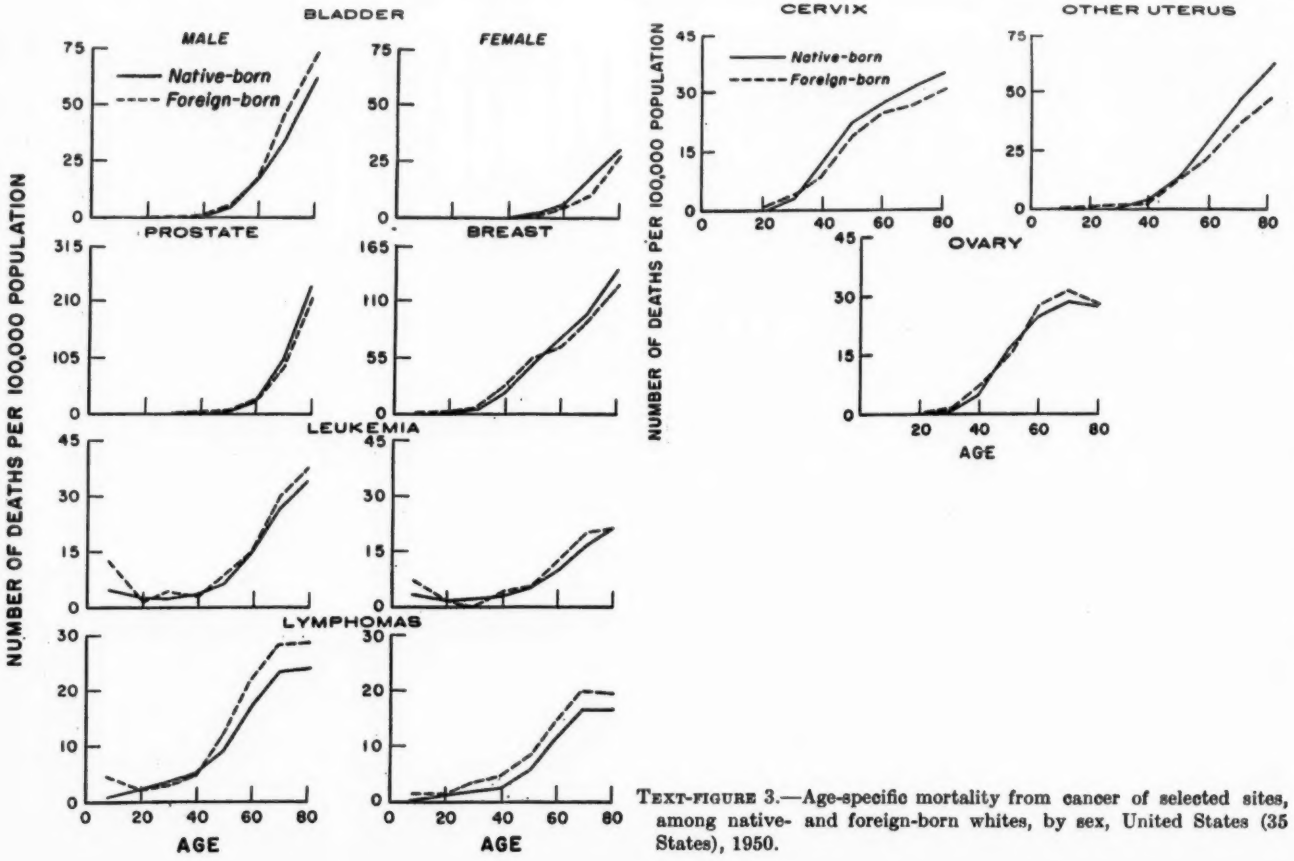


TEXT-FIGURE 2.—Age-specific mortality from cancer, all sites, among native- and foreign-born whites, by sex, United States (35 States), 1950.


BUCCAL CAVITY AND PHARYNX
MALE
FEMALE
Native-born
Foreign-born
DIGESTIVE SYSTEM
ESOPHAGUS
STOMACH
NUMBER OF DEATHS PER 100,000 POPULATION
AGE


INTESTINES
MALE
FEMALE
Native-born
Foreign-born
RECTUM
PANCREAS
LUNG AND BRONCHUS
NUMBER OF DEATHS PER 100,000 POPULATION
AGE


BLADDER
CERVIX
OTHER UTERUS



TEXT-FIGURE 3.—Age-specific mortality from cancer of selected sites, among native- and foreign-born whites, by sex, United States (35 States), 1950.

TABLE 2.—Summary of earlier findings on mortality from cancer, all sites, by nativity

Source	Locality	Nature of findings
Davis (*6*)	Boston, Massachusetts (1900)	Age-adjusted mortality for white males whose mothers were born abroad was 145 percent higher than for males of mothers born in the United States. The corresponding excess for females was 9 percent. Irish were the predominant ethnic group.
Dublin (*4*)	New York and Pennsylvania (1910)	Data for 6 foreign-born groups indicated that age-adjusted mortality for foreign-white males ranged 40 to 50 percent higher than for native-white males. The corresponding excess risk for foreign-white females was much smaller, 5 to 10 percent.
Lombard and Doering (*8*)	Massachusetts (1920)	Age-adjusted mortality for foreign-born males was 88 percent higher than for native-white males of native parentage; contrast of the foreign-born with all native-white males reduced the excess risk of 38 percent. The corresponding differentials for females were 20 and 8 percent, respectively.
New York State Department of Health (*26*)	New York State, exclusive of New York City (1929–1931)	Age-adjusted mortality for foreign-born males was 73 percent higher than for native-white males of native parentage; contrast with all native-white males reduced the excess risk to 27 percent. The corresponding differentials for females were 26 and 1 percent, respectively.

New York City experience (55% of the 1950 native-white population over 35 years in the New York City metropolitan area was of foreign or mixed parentage), where MacMahon (*13*) noted only a trivial difference between native and foreign whites in the 1955 cancer death rates.

The relationship of the native- and foreign-born rates varied markedly by site. In the present material, sites with sizable excess risks among the foreign-born included esophagus, stomach, and lung. The experience for upper digestive tract contrasted sharply with the much closer correspondence in rates for intestine and rectum. Buccal cavity, ovary, prostate, bladder (males), and leukemia were sites exhibiting less pronounced nativity group differences. Significant deficits were noted among foreign-born women for bladder, breast, and uterus. The age-specific rates for breast presented an unusual configuration, foreign women having the higher rates at younger ages with a distinct reversal after age 50; the consistency of this feature with other pieces of epidemiological evidence is discussed later in the text. The deficit for female reproductive organs among the foreign-born tended to counterbalance their higher risks for other sites and to minimize the female nativity differential for all sites. When comparisons were restricted to nonreproductive organs, males still displayed the greater nativity differentials, but the sex disparity in this regard was reduced.

The relationships described for esophagus and stomach, intestine and rectum, breast, and uterus can be seen in the earlier Massachusetts (*8*)

and New York data (*26*). Similar patterns in native- and foreign-born mortality appeared for most sites in the New York City 1955 results (*13*). The one important exception in the latter figures, absence of a lower rate among the foreign-born for cervix and corpus uteri, may be related to the presence of a large native-born population of foreign parentage in New York City.

Ethnic Groups

Table 3 summarizes the information on cancer mortality by site for whites born in 12 foreign countries. While Mexicans are classified as whites in vital statistics and census returns, the designation is not strictly accurate, since biologically they represent a mixture of white and Indian blood. The data for Mexicans should be reviewed in the context of findings for American Indians as well as those for native whites and other migrant groups.

Since regional differences in mortality prevail for certain sites, it may be worth re-emphasizing that the SMR's have been adjusted for regional distribution of the foreign-born population in addition to age. The observed and expected numbers of deaths and the derivative SMR's are included in the appendix for five regions (*see* text-fig. 1). The regional data leave little doubt that the ethnic-group effects noted in the national summary persisted throughout the country and would rule out the possibility that they could have been produced solely through confounding with other factors contributing to regional variation. This strengthens the inferences which may be drawn from these data.

In text-figures 4 to 6 the SMR's are rearranged in order of magnitude. It is immediately apparent that the two sexes differed in many particulars on the ranking of ethnic groups for all sites and individual sites. Although the relative rankings would not normally be affected, the reader should always bear in mind that the U.S. native-white experience has been taken as the standard of reference. When the U.S. rate for a given site is low compared to those reported elsewhere, the corresponding SMR's for migrants and other populations will read high, even though it is this country which has the aberrant rate.

For all sites combined, males in 10 of the 12 foreign-born groups considered had significantly higher SMR's (at the 1% level) than native whites; migrants from Poland, Ireland, Czechoslovakia, and USSR were the groups with greatest risks. The spread in the female SMR's was not so pronounced and when the test criterion used for males was applied, women from 3 countries (USSR, Mexico, Ireland) had significantly higher ratios and 1 (Italy) a lower ratio than the native whites. Eliminating reproductive organs from consideration would accentuate the variation among the female groups, but would not raise it to the level prevailing among males.

The 1950 data on ethnic-group risks for all sites conformed closely to earlier findings. Davis (*6*) remarked on the extremely high cancer mortality in Boston as of 1900 among those of Irish descent (persons whose

TABLE 3.—Standardized cancer mortality*† (SMR=100 for native-born whites) with number of deaths observed for foreign-born whites by country of birth for selected primary sites, by sex, United States (35 States), 1950

Site (International List Numbers, sixth revision, 1948)	Total foreign-born white	Total 12 countries	England and Wales	Ireland	Norway	Sweden	Germany	Austria	Czechoslovakia	Poland	USSR	Italy	Canada	Mexico
MALES														
All sites (140–205)														
Observed	20201	15492	1114	928	515	899	2428	938	630	2129	1970	2671	1052	218
SMR	124‡	120‡	118‡	134‡	114‡	109‡	122‡	119‡	133‡	145‡	130‡	103	114‡	91
All sites (excluding reproductive organs 170, 177–179)														
Observed	18319	13963	981	810	434	749	2114	867	579	1999	1853	2480	898	199
SMR	130‡	125‡	122‡	136‡	114‡	109§	126‡	126‡	142‡	154‡	138‡	108‡	114‡	94
Buccal cavity and pharynx (140–148)														
Observed	694	552	45	64	24	19	75	25	26	72	25	122	52	3
SMR	105	105	118	224‡	134	59§	93	76	138	119	39‡	112	145‡	34§
Digestive system (150–159)														
Observed	9958	7717	545	473	249	458	1265	458	347	1031	969	1335	482	105
SMR	145‡	142‡	136‡	162‡	127‡	129‡	148‡	138‡	171‡	166‡	152‡	124‡	125‡	115
Esophagus (150)														
Observed	834	656	32	53	22	29	90	39	37	131	61	126	30	6
SMR	214‡	211‡	150§	315‡	239‡	167‡	198‡	196‡	322‡	336‡	158‡	189‡	147§	146
Stomach (151)														
Observed	3750	2821	190	131	121	210	491	166	139	413	353	388	167	52
SMR	179‡	170‡	151‡	149‡	189‡	181‡	182‡	168‡	222‡	225‡	187‡	123‡	138‡	177‡
Intestines (152–153)														
Observed	2074	1661	122	128	38	96	260	93	70	173	233	314	122	12
SMR	115‡	115‡	117	161‡	76	105	114§	104	130§	104	137‡	109	121§	58
Rectum (154)														
Observed	1331	1042	75	82	29	42	185	65	44	111	121	217	65	6
SMR	128‡	125‡	125§	174‡	109	86	148‡	123	150‡	115	119	124‡	115	48
Pancreas (157)														
Observed	998	793	81	50	24	43	113	49	21	106	108	137	48	13
SMR	120‡	122‡	168‡	148‡	100	101	114	124	87	143‡	140‡	106	104	96

Lung and bronchus (162–163)														
Observed	3115	2249	160	79	37	83	264	168	84	434	380	382	130	48
SMR	133‡	122‡	131‡	81	70§	90	112	140‡	134‡	188‡	155‡	90§	109	122
Prostate (177)														
Observed	1767	1438	128	113	81	145	286	65	49	125	111	173	144	18
SMR	89‡	90‡	97	127§	120	114	96	72‡	80	79‡	68‡	64‡	115	80
Bladder (181)														
Observed	812	624	52	30	22	35	112	35	21	69	87	106	44	11
SMR	99	94	105	81	101	87	109	86	90	91	111	79§	96	111
Leukemia (204)														
Observed	621	476	27	24	17	32	69	23	24	56	75	92	32	5
SMR	107	106	81	106	102	108	101	88	144	108	145‡	106	94	45
Lymphomas (200–203, 205)														
Observed	682	510	27	22	16	24	67	40	18	72	78	106	37	3
SMR	116‡	113‡	85	94	105	90	106	147§	111	135§	141‡	113	109	23‡
All other sites														
Observed	1975	1501	101	85	62	89	213	98	46	207	210	269	101	20
SMR	104	101	94	111	118	94	94	111	84	122‡	121‡	92	92	59§
FEMALES														
All sites (140–205)														
Observed	14262	11485	945	925	392	663	1983	659	446	1258	1481	1384	1095	254
SMR	106‡	105‡	107§	110‡	111§	102	106§	104	106	105	123‡	85‡	107§	122‡
All sites (excluding reproductive organs 170–176)														
Observed	9696	7810	598	633	266	459	1349	446	314	878	1093	928	681	165
SMR	119‡	118‡	109§	123‡	119‡	109	113‡	120‡	123‡	126‡	154‡	98	110§	142‡
Buccal cavity and pharynx (140–148)														
Observed	121	96	5	15	2	11	9	7	3	10	8	10	15	1
SMR	90	89	57	181§	57	164	47§	111	71	88	67	62	153	63

See footnotes at end of table.

TABLE 3.—Standardized cancer mortality*† (SMR=100 for native-born whites) with number of deaths observed for foreign-born whites by country of birth for selected primary sites, by sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Total foreign-born white	Total 12 countries	England and Wales	Ireland	Norway	Sweden	Germany	Austria	Czechoslovakia	Poland	USSR	Italy	Canada	Mexico
FEMALES—Continued														
Digestive system (150–159)														
Observed	6505	5258	403	455	182	307	951	295	226	572	694	632	438	103
SMR	128‡	126‡	117‡	137‡	128‡	114§	125‡	127‡	142‡	132‡	158‡	107	114‡	156‡
Esophagus (150)														
Observed	218	154	15	15	4	10	25	2	6	12	42	9	13	1
SMR	211‡	185‡	221‡	205‡	167	200§	163§	43	222§	138	442‡	73	181§	91
Stomach (151)														
Observed	2012	1608	125	140	75	107	315	85	86	176	182	176	107	34
SMR	190‡	186‡	169‡	204‡	236‡	175‡	189‡	185‡	264‡	209‡	210‡	153‡	130‡	238‡
Intestines (152–153)														
Observed	1883	1532	133	145	38	74	291	95	53	142	219	166	162	14
SMR	98	97	103	113	74	76§	102	105	89	85§	128‡	72‡	113	60§
Rectum (154)														
Observed	713	588	41	59	15	25	95	33	26	75	74	75	58	12
SMR	104	105	90	129§	86	75	97	102	127	125	120	89	114	136
Pancreas														
Observed	660	538	34	48	21	40	73	39	22	62	71	69	46	13
SMR	129‡	130‡	96	150‡	144	145§	98	170‡	138	144‡	163‡	119	119	169
Lung and bronchus (162–163)														
Observed	573	476	41	33	19	22	73	27	14	54	96	51	26	20
SMR	139‡	142‡	150‡	131	168§	108	126§	141	109	150‡	249‡	106	84	313‡
Breast (170)														
Observed	2249	1809	178	162	63	105	336	100	63	168	198	206	210	20
SMR	83‡	83‡	105	97	97	90	96	77‡	77§	68‡	78‡	60‡	105	45‡
Female genital organs (171–176)														
Observed	2317	1866	169	130	63	99	298	113	69	212	190	250	204	69
SMR	88‡	88‡	102	84‡	99	87	88§	89	83	86§	78‡	76‡	103	150‡

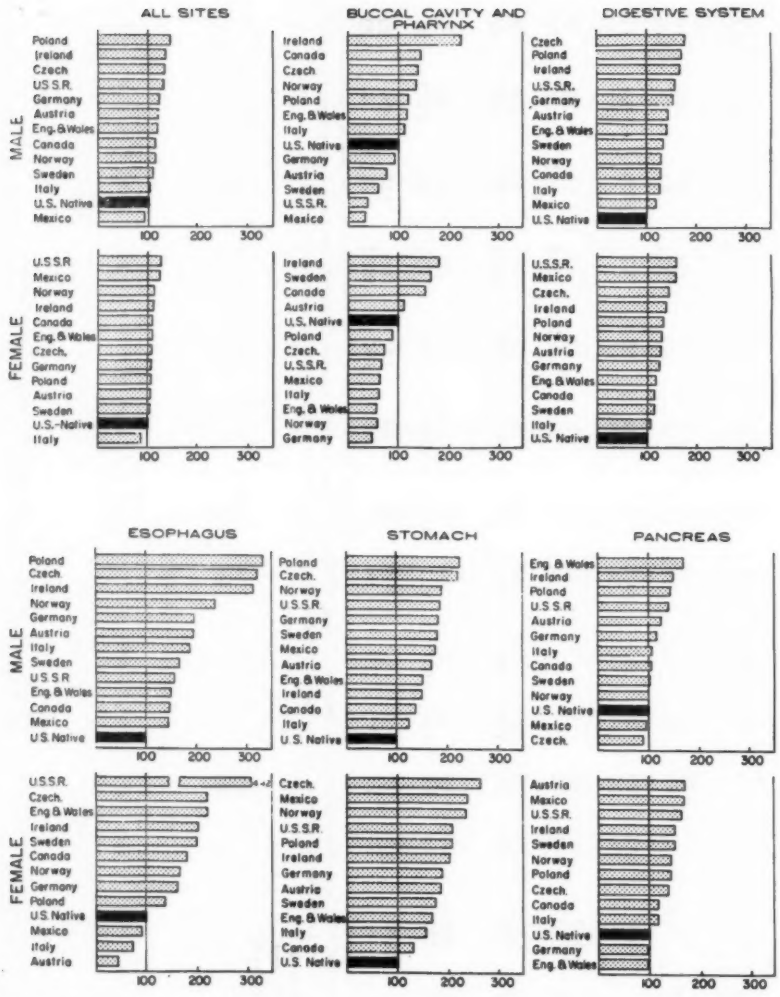
Cervix (171)														
Observed	727	587	58	33	18	25	98	30	25	71	37	78	73	41
SMR	90‡	92§	115	73	98	78	101	78	100	95	50‡	77§	116	241‡
Uterus, other and unspecified (172–173)														
Observed	751	613	57	48	21	39	93	29	27	74	49	97	63	16
SMR	78‡	79‡	93	83	86	86	71‡	63§	86	83	57‡	84	89	120
Ovary (175)														
Observed	733	579	42	39	21	31	91	45	14	63	97	65	60	11
SMR	94	94	90	86	118	100	98	117	59§	85	130§	64‡	107	76
Bladder (181)														
Observed	229	179	19	15	6	6	42	14	2	12	16	19	25	3
SMR	67‡	65‡	79	65	64	33‡	80	92	20§	42‡	55§	49‡	97	77
Leukemia (204)														
Observed	398	315	19	18	10	19	44	15	9	48	57	41	32	3
SMR	112§	111	81	85	109	121	91	93	84	157‡	182‡	97	113	47
Lymphomas (200–203, 205)														
Observed	431	351	26	18	11	16	53	25	12	49	56	41	38	6
SMR	123‡	127‡	119	93	124	102	121	152§	108	152‡	177‡	96	140§	95
All Other Sites														
Observed	1368	1088	78	76	34	71	168	62	46	127	166	125	106	29
SMR	97	97	83	92	89	102	86	97	104	104	136‡	76‡	97	122

*The standardized mortality ratios are expressed, after adjustment for age by the indirect method, in terms of the corresponding cancer death rates for native-born whites; the SMR's are based on the summation of observed and expected numbers of deaths in each region and are thus adjusted for the regional distributions of the foreign-born groups.

†For regional grouping of States, *see* text-figure 1.

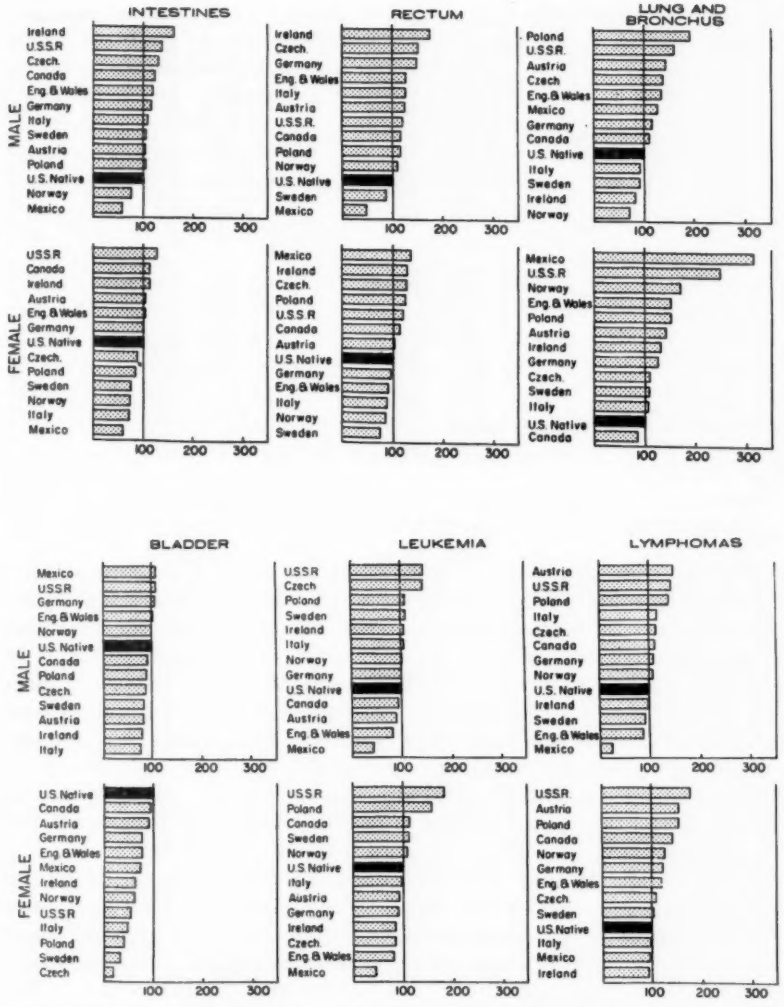
‡SMR significantly different from 100 at 1% level.

§SMR significantly different from 100 at 5% level.


ALL SITES
BUCCAL CAVITY AND PHARYNX
DIGESTIVE SYSTEM
MALE
Poland Ireland Czech U.S.S.R. Germany Austria Eng. & Wales Canada Norway Sweden Italy U.S. Native Mexico
Ireland Canada Czech Norway Poland Eng. & Wales Italy U.S. Native Germany Austria Sweden U.S.S.R. Mexico
Czech Poland Ireland U.S.S.R. Germany Austria Eng. & Wales Sweden Norway Canada Italy Mexico U.S. Native
FEMALE
U.S.S.R Mexico Norway Ireland Canada Eng. & Wales Czech, Germany Poland Austria Sweden U.S.-Native Italy
Ireland Sweden Canada Austria U.S. Native Poland Czech. U.S.S.R. Mexico Italy Eng. & Wales Norway Germany
U.S.S.R. Mexico Czech. Ireland Poland Norway Austria Germany Eng. & Wales Canada Sweden Italy U.S. Native
100 200 300
ESOPHAGUS
STOMACH
PANCREAS
MALE
Poland Czech. Ireland Norway Germany Austria Italy Sweden U.S.S.R. Eng. & Wales Canada Mexico U.S. Native
Poland Czech. Norway U.S.S.R. Germany Sweden Mexico Austria Eng. & Wales Ireland Canada Italy U.S. Native
Eng. & Wales Ireland Poland U.S.S.R. Austria Germany Italy Canada Sweden Norway U.S. Native Mexico Czech.
FEMALE
U.S.S.R. Czech. Eng. & Wales Ireland Sweden Canada Norway Germany Poland U.S. Native Mexico Italy Austria
Czech. Mexico Norway U.S.S.R. Poland Ireland Germany Austria Sweden Eng. & Wales Italy Canada U.S. Native
Austria Mexico U.S.S.R. Ireland Sweden Norway Poland Czech. Canada Italy U.S. Native Germany Eng. & Wales
100 200 300

TEXT-FIGURE 4.—Standardized cancer mortality ratios arranged in order of magnitude by country of birth and by sex for selected sites, United States (35 States), 1950.

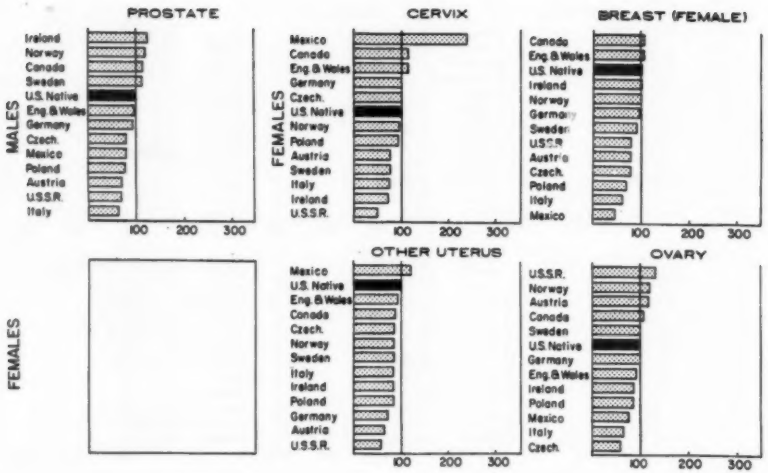
mothers were born in Ireland), particularly among males, where they constituted the predominant foreign stock at the time. Later the Bureau of the Census (*27*) tabulated cancer deaths occurring in 1920 among the foreign-born and those of foreign parentage in 2 States (New York and Pennsylvania) and 3 cities (Chicago, New York, and Philadelphia). The Italians and their immediate descendants clearly showed the lowest mortality of the foreign stock in these areas for both sexes, their age-adjusted rates being as low or lower than those for whites of native parentage.



TEXT-FIGURE 5.—Standardized cancer mortality ratios arranged in order of magnitude by country of birth and by sex for selected sites, United States (35 States), 1950.

The highest rates were experienced by the Irish of both sexes, closely followed by the Germans (including those from German Poland). Scandinavians and migrants from England, Scotland, and Wales occupied an intermediate position.

Dublin's (*4*) ranking of males according to cancer mortality for the 5 foreign-born groups considered in his analysis of the New York State vital statistics (1910)—Russians, Irish, Germans, English, Italians—matched the present data almost exactly; the Russian population in 1910 included



TEXT-FIGURE 6.—Standardized cancer mortality ratios arranged in order of magnitude by country of birth and by sex for selected sites, United States (35 States), 1950.

Poles who were classified separately in 1950. While the agreement was not so precise for females, the features of a high Irish and a low Italian rate stood out in Dublin's tables. In the unpublished data for New York State, exclusive of New York City, 1928–32, the cancer death rates by country of birth were computed only for both sexes combined. Since the ranking of ethnic groups differs by sex, this undoubtedly blurred some of the distinctions noted in other sources. Nevertheless, the familiar pattern of a high cancer risk among the Irish and a low risk among the Italians emerged. This material also indicated a high mortality among Russians, and the sex distribution of deaths indicated that the excess risk among females was a finding in accord with the present data.

Lombard and Doering (*8*) re-examined cancer mortality in Boston for several years around 1920, by which time other foreign-born had joined the Irish in that city. Their results indicated that males born in Ireland had the highest cancer death rates, followed by Teutons (from Germany, Austria, Belgium, and Holland), and Italians had the lowest rate. The order differed somewhat among females; those born in Canada and England exhibited higher rates than women from Ireland. Italian women placed lowest on the scale.

The more striking ethnic-group differences for all forms of cancer, such as those between Italians on one hand and Irish males and Russian females on the other, may still prevail among the foreign-born and their immediate descendants. Some evidence of this can be detected in the New York City death rates for 1955, in which MacMahon (*13*) classified the population and deaths by religious affiliation and observed that Catholic women had substantially lower cancer mortality than Jewish women. Most of the Jewish women or their parents had migrated from Russia, and in New York

City a classification by religion or country of birth would be almost interchangeable for that group, so that his finding of a high rate for Jewish women would seem consistent with other reports on cancer mortality among Russians. Italians constitute one of the major Catholic groups in New York City and a low cancer risk for these women could well have depressed the over-all rate for Catholic women. Similar religious group differentials for males did not appear in MacMahon's data.

Site

The impression gained from inspection of table 3 and text-figure 3 is that no ethnic group had uniformly high or low rates for all sites considered. Rather, each group presented its own specific pattern of excesses and deficits. While some apparent departures from prevailing experience will arise by chance if enough comparisons are made within a single set of data, the number of such deviations is too large here to entertain this explanation. Furthermore, the present results may be shown to be generally consistent with several other independent observations. The more impressive departures from the experience of the native-white population uncovered by this study, with due note of the numbers of deaths involved, may be summarized as follows:

Site	Excess	Deficit
Buccal cavity and pharynx	Ireland	USSR (males)
Esophagus	Poland (males)	
	Czechoslovakia	
	Ireland	
	USSR (females)	
Stomach	Poland	
	Czechoslovakia	
	Norway	
	USSR	
Intestines	Ireland (males)	Mexico
Rectum	Ireland (males)	Mexico (males)
Bronchus and lung	Poland	
	USSR	
	Mexico (females)	
Female breast		Mexico
		Italy
		Poland
Cervix uteri	Mexico	USSR
Bladder		Sweden (females)
		Poland (females)
		Italy (females)
Leukemia	USSR	
Lymphomas	USSR	

Buccal Cavity and Pharynx

The high buccal-cavity risks among the Irish corroborated Lombard and Doering's report on the distribution of buccal-cavity and esophageal deaths in Boston, though these authors detected no excess risk among

Canadians, most of whom would have been French Canadians in the Boston area (*8*). In the present series, the Irish excess was most pronounced for tongue (33 deaths observed versus 9.5 expected), but was present for all other detailed sites within this category.

No one site within the buccal cavity could be singled out as predominantly responsible for the low risk among USSR males. The results for this group were consistent with the Boston experience for Russians and Poles. With the large Jewish component among the Russian-born in the United States [at the time of the 1940 census, Yiddish was the mother tongue of 61.9% of USSR migrants and 17.6% of Polish migrants (*28*)] taken into account, the results are also supported by the recent New York City data, indicating the mortality among Jewish males to be well below that for other groups (*13*).

The conjunction of a low male and high female SMR for Swedish migrants and the resulting low sex ratio of 1.7:1 closely resemble the situation in Sweden, where an apparent association of buccal and pharyngeal cancer in women with Plummer-Vinson disease has been commented on by Ahlbom (*29*) and others.

Esophagus

Perhaps the most prominent epidemiologic feature of esophageal cancer is the extreme varibility in sex ratio apparent in the incidence and mortality data collected from many countries. The same experience was apparent in the U.S. migrant groups. While some of the observed international variation might be due to different practices in reporting and assignment to esophagus of post-cricoid and cardia lesions, which have their own characteristic sex ratios, effects of this sort should be minimized in the data from any one country. The U.S. ethnic-group data would tend to cast doubt on the possibility of accounting for an important part of the sex-ratio variation in this manner.

All the foreign-born males had substantially greater esophageal rates than the native whites; the highest SMR's were observed among males from Poland, Czechoslovakia, and Ireland. The rankings by country of birth were quite different among females, with women from USSR having an SMR far above that of any other group. Migrants from Czechoslovakia and Ireland showed high rates for both sexes.

As indicated in the preceding section, the Irish experience agreed with the Boston findings for buccal cavity and esophagus (*8*). The two sources were in essential agreement on the high rate for Russian women, particularly when one remembers that the Boston figure must have been diluted by the inclusion of lower-risk Polish women. The sex disparity in mortality for USSR migrants had its counterpart in the New York City 1955 data, which indicated Jewish males to have a low, and Jewish females a high rate for this site (*13*). While no USSR vital statistics were available for direct comparison with the low male-female ratio among USSR migrants, 1.5:1, a low sex ratio may prevail there. For example, among admissions to the Leningrad Institute of Oncology, the sex ratio

for diagnosed cases of esophagus was 2.5 : 1 (*30*). In adjacent Finland the low sex ratio due to high female rates has been well documented (*31*, *32*), and it is conceivable that similar relationships may exist in other East European countries. Because of the representation of Jews among USSR migrants, the depressed sex ratio for esophagus death rates in Israel (*25*) is interesting and suggestive.

The relatively small number of observations would caution against attaching too much weight to the high sex ratio for Austrian migrants, though it may be remarked that in this attribute Austria ranked third in an international compilation, following France and Switzerland (*25*).

Stomach

The data for stomach resembled those for the esophagus in that all ethnic groups had stomach-cancer rates well above those for native whites. However, the sex correspondence on the rankings by country of origin was much closer for stomach, which fitted the pattern of relatively stable sex ratios for this site established by observations on a wide variety of groups (*19*, *33*, *34*), irrespective of the absolute magnitude of the incidence and mortality rates.

The highest risks were found for migrants from Poland, Czechoslovakia, Norway, and USSR. While the Irish had above-average risks, they did not stand out in this respect as they did for other sites within the digestive tract. The rates for Italians of both sexes were comparatively low. One may be reasonably certain that the distribution of SMR's has not been grossly affected by overcertification of stomach as opposed to large intestine in some groups, since essentially the same type of relationship can be discerned in the results for total digestive system.

The collateral evidence for an excess stomach-cancer rate among the foreign-born in the United States has been reviewed elsewhere (*19*). The Boston data were generally consistent on the relative standing of ethnic groups (*8*). The New York City results, which indicated Jews had higher rates than Catholics of either sex, did not contradict the impression of high rates for persons from USSR and low rates for those from Italy (*13*).

In the countries of origin that had vital statistics data available for comparison, the experience of their migrants resembled that of the parent populations; the nature of these relationships is sketched in further detail in a following section. In the USSR where comprehensive mortality data are lacking, recent regional cancer surveys emphasize the very high stomach-cancer rates experienced there (*35*, *36*). On the other hand, the presumably high rates for Jews in the United States were not reflected in the low Israeli stomach-cancer death rates (*25*).

Intestines and Rectum

The lower digestive tract was characterized by a reduced scale of variation among ethnic groups compared to that encountered for esopha-

gus and stomach. The larger SMR's among Irish males for intestine and rectum fitted into a larger pattern of excess mortality throughout the digestive tract, most noticeable for sites other than stomach, and warrant further investigation. The Boston data had suggested only an above-average risk typical of other foreign-born groups for intestinal cancer among the Irish (*8*).

A low risk among Mexicans was generally indicated and the one exception, the SMR for female rectum, was not statistically significant. The depressed relative frequency of these sites for Mexicans in the Los Angeles County Hospital necropsy series reported by Steiner (*1*) would appear consistent with the present results. Mexicans are a racially heterogeneous people with a high proportion of Indian blood, and the close resemblance of their rates for these two sites to those reported for the American Indians is provocative (*37*).

The picture portrayed by international comparisons differed materially from that to be inferred from the ethnic-group data. Instead of the more compressed range of SMR's by ethnic group, the international variation in mortality from cancer of the intestines and rectum was as great as for other sites within the digestive tract. Also, the United States ranked in the top half of the countries reporting on mortality from these sites (*25*), and this may offer difficulties in a reconciliation with the somewhat higher rates experienced by the foreign-born in this country.

Pancreas

Although mortality from cancer of the pancreas is slightly greater among foreign whites, from these data it would be difficult to designate with confidence ethnic groups experiencing elevated risks. Since this disease presents formidable diagnostic uncertainties, it has never been regarded as a promising subject for epidemiologic investigation. Consequently, there are virtually no other background data against which these findings may be assessed. The possibility that USSR migrants have an above-average rate should not be dismissed, since Jews of both sexes in New York City were described as subject to higher risks for this site (*13*).

The mortality in the United States for whites and nonwhites was among the highest recorded anywhere (*25*). In view of this, why the foreign-born should have rates exceeding both those for the U.S. native-born and for populations in the respective countries of origin is puzzling. Possibly, pancreatic cancer is diagnosed more frequently here than abroad.

Lung and Bronchus

In contrast to most sites, the ethnic variation for lung and bronchus appeared about as marked for female as for males, though this may merely reflect greater sampling fluctuations due to fewer female deaths. The highest male rates occurred among Polish and Russian migrants; these and the remainder of the ranking by countries of origin coincided almost

exactly with Mancuso's results from Cleveland, Ohio (*11*). For females the distinctive features were the excess among the Mexican and USSR groups. The Polish and Russian SMR's provided an interesting example of sex differences in risk, the spread in female rates departing noticeably from the pattern set among males.

The suggestion of a higher risk for Jewish women in these ethnic-group data would appear consistent with the high female lung-cancer mortality recorded in Israel (*25*). In New York City the 1955 rates for Jewish males were somewhat lower and for Jewish females only slightly higher than for other religious groups (*13*). This raises the question as to what part of the excess USSR risks might be attributable to the concentration of this group in New York City and certain other metropolitan areas. The more broadly based data for six Standard Metropolitan Areas, discussed later, would suggest that the risk for USSR females remains high even after place of residence is controlled in the comparisons.

The excess for Mexican females was first suspected by Steiner on the basis of an unusually low sex ratio for this group in a Los Angeles necropsy series (*1*). A review of the 1950 death certificates in California by Buechley and others confirmed this supposition (*14*). While the 1950 California observations have been incorporated in the present material, it may be mentioned that the experience from the other States yielded substantially the same results for Mexican women. Since many Mexicans have Indian blood, the depressed sex ratio for Indians (*37*) is worth noting in this context, though the Indian ratio derives from a low rate among males rather than an elevated one among females.

Since the outstandingly high ethnic-group risks occurred for countries with no available vital statistics, not too much can be said about the consistency of ethnic-group and international comparisons. The below-average SMR's for males migrating from Italy, Norway, and Sweden did agree essentially with the position of these countries in international rankings (*25*).

Breast

Contrary to the experience for many other sites, the native-white breast-cancer mortality virtually equaled the highest foreign-born rates. The differences between the native whites and migrants from Canada, the British Isles, and several West European countries were trivial and not significant. The sharply reduced risks among women from Italy and Poland were foreshadowed by the earlier Boston findings (*8*). While there is no reliable information from Poland, the mortality for Italian migrants agreed rather well with that recorded in Italy (*25*). The Mexican breast cancer SMR would appear to confirm Steiner's deduction from a Los Angeles necropsy series that this lesion occurs less frequently in this group (*1*). Pursuing the comparison of Mexicans and Indians, we find that the low Mexican rate resembled rather closely the Indian experience (*37*).

Uterus

Despite the well-known differences in epidemiologic characteristics of cervix and corpus uteri, the sometimes imprecise statement of detailed anatomical site in medical certifications, which leads to the classification of some cervical cancer deaths as "other and unspecified uterus," requires that uterine cancer mortality be reviewed as a unit. Bearing this precaution in mind, one may conclude that the prominent features were the low USSR and high Mexican SMR's for cervical cancer, and possibly a below-average cervical risk among Italian migrants. Uncertainties in the basic data preclude firm conclusions about "other uterus." Lower Russian and Italian uterine cancer rates were reflected in the Boston experience (*8*). The finding for Russian women could certainly have been anticipated from the literature on the infrequent occurrence of cervical carcinoma in Jews (*38–42*).

The cervix rate for women from Mexico was in complete accord with the excess relative frequency noted in necropsy data (*1*) and consistent with the exceptionally high mortality for this site reported for American Indians (*37*).

The decreased cervical risk for Italian migrants, if confirmed, would be a most interesting phenomenon, a group with low risk for both cervix and breast, running counter to the usual inverse relationship in risks for these two sites. The pattern revealed by the foreign-born data should be accepted with reservation, since a conventional inverse relationship for breast and uterus is indicated in the vital statistics for Italy (*25*). The USSR migrants might constitute another possible exception to this rule of inverse relationship in risk.

Ovary

The ethnic-group distribution for ovarian cancer revealed no striking departures from the native-white experience. The one SMR which differed significantly, when tested at the 1 percent level, was for Italian women. The low rate in this group did not disagree with the experience in Italy, where mortality recorded for this site was only a fraction of the rate in most West European countries (*25*).

Prostate

While the present data suggested a measure of ethnic-group variation in prostatic cancer similar to that observed for ovary, they can be regarded only as suggestive, in the absence of collateral information from other studies of mortality among the foreign-born. Reliance on mortality rather than incidence data may have some advantage for this site, since the former presumably represented clinically active cases and not merely lesions uncovered by histologic examination of operative specimens. Low risks among migrants from Italy and USSR remain topics for further investigation. The New York City data point to a lower Jewish risk, consistent with the USSR rate (*13*). Internationally, Italy and Israel report among the lowest death rates for this site (*25*).

Bladder

Foreign-born males exhibited no important variation in bladder cancer in sharp contrast to the wide range in female rates, which is contrary to the customary picture of greater variability among males. Admittedly, the observations on women need to be augmented. Nevertheless, some female ethnic-group rates (Czechoslovakia, Sweden, Poland, Italy, and USSR) were so low that one must anticipate that one or more of them will be confirmed by more extensive observations. The results for women were also unusual in indicating that native whites had the highest rates.

Mancuso's study of bladder cancer in Ohio (*11*) covered only 39 deaths among foreign-born males and did not detect, nor could it have been expected to do so, any significant ethnic-group differences. Whether the New York City data (*13*) supported the present findings is conjectural, though the lower risk for Jewish females there might be described as a possibly consistent feature.

The deficits for foreign-born women in the United States ran somewhat counter to the international comparisons, which revealed only slight differences between the United States and most other countries in female mortality from cancer of the urinary organs, though it was true that Ireland, Italy, and Israel had female rates substantially below that prevailing in this country (*24*). Since the latter three countries had low uterine-cancer rates as well, some area of uncertainty may remain concerning the effect of diagnostic practices on international comparisons for sites within the pelvis. If it could be established that female migrants generally experience bladder-cancer risks lower than those for native whites and residents in the respective countries of origin, this would pose several provocative questions worthy of further study.

Leukemia

The most firmly established finding for leukemia (*see* table 3) would be the excess risk for male and female migrants from the USSR. This fitted well with the New York City report of high mortality from this disease among Jews (*13*). In an earlier investigation of diagnosed cases in Brooklyn, MacMahon and Koller (*12*) noted that the excess among Jews was manifest in each of the three common varieties: acute myeloid, chronic myeloid, and chronic lymphatic. While the finer diagnostic details are not so precisely stated on death certificates, the present mortality data also indicated that the leukemia excess among Russian migrants was not confined to any one category. The high USSR group risk within the United States is in keeping with the elevated leukemia mortality recorded in Israel, particularly for females (*25*).

Low risks among Mexicans for both leukemia and lymphomas were suggested, which coincided with the relative infrequency of these diagnoses in the Los Angeles necropsy series (*1*) and the depressed mortality rates among Indians (*37*).

Lymphomas

Three migrant groups, USSR, Austria, and Poland, had above-average lymphoma risks for both sexes. The epidemiologic literature on these diseases is not extensive, since most of the published papers have dealt with Hodgkin's disease (*43–45*), and collection of additional data should be encouraged, including further study of ethnic-group variation. MacMahon's review of Hodgkin's disease among Brooklyn residents (*43*) led him to conclude that it appeared as a cause of death "about one and a half times more frequently among Jewish than among non-Jewish patients," which could be considered consistent with the USSR risks noted here. The later New York City study (*13*) revealed the Jewish excess to appear in all the lymphoma categories—Hodgkin's disease, lymphosarcoma, reticulum-cell sarcoma, multiple myeloma—and the same tendency was evident in the national data for USSR migrants.

Standard Metropolitan Areas

Since the foreign-born are concentrated in large urban centers, it is natural to inquire as to what part of the ethnic-group variations in risk in the over-all national presentation of table 3 might be accounted for, if separate schedules of rates were introduced for urban and rural populations. This statement should not be interpreted as necessarily implying that the foreign-born would be affected by environmental factors in a manner common to all urban populations. The relationship between ethnic group and urban residence may be more complex. Lombard and Doering (*46*) in a study in Massachusetts, 1921–25, noted that the cancer death rates for the foreign-born increased with size of community, while those for the native-born of native parentage remained essentially unchanged. Whether these earlier results apply to more recent experience in Massachusetts, or more generally to other areas, is not known.

Unfortunately, the material in table 3 cannot be broken down into separate urban and rural components with SMR's based on the native-white experience in each population class. The Bureau of the Census, in its data distributing the foreign-born groups by residence, used the 1950 "new" urban definition including the urban fringes. This was incompatible with the residence allocation of death certificates, which must follow political subdivision lines rather than economic or trade area boundaries.

For this reason, we examined the data for six large Standard Metropolitan Areas (New York-northeastern New Jersey, Chicago, Cleveland, Detroit, Los Angeles, and San Francisco-Oakland) for which age-sex distributions of the ethnic-group populations have been published. Our presentation took two forms: First, SMR's were computed with the rates for total whites (native- and foreign-born) in each area as the standard for determining the expected number of deaths, and the latter were then cumulated. The basic mortality rates were a by-product of a systematic examination of mortality-by-cause in the economic subregions and Stand-

ard Metropolitan Areas of the United States, 1949–51 (*47*). Reference to the rates for all whites provided a more severe test than would be afforded by the experience of native whites alone. If the SMR's thus computed still deviated markedly from the over-all white experience, one could be reasonably certain that the ethnic-group deviations were not ascribable to common factors affecting the white metropolitan populations.

The second approach segregated the six Standard Metropolitan Areas from the remainder of the three regions (Northeast, North Central, West) in which they were located. The regional native-white rates were used to calculate the expected numbers of deaths both inside and outside the Standard Metropolitan Areas. The expected and observed numbers for the three regions were consolidated in table 5. The purpose of this table was merely to determine whether the effects noted in the composite national data tended to persist in the two parts of the regions. Fine differences arising from contrasts of these SMR's should not be relied on as indicators of true differences in risk between the foreign-born living in the metropolitan centers and elsewhere, since some of the lower SMR's outside the metropolitan areas could reflect the influence of diagnostic standards and related factors.

Before we review tables 4 and 5, four general remarks are in order: (*a*) Since many of the effects in table 3 were specific to certain sites and ethnic groups, one would not expect all of them to disappear with control for residence. There obviously could be no common factors associated with urban environment for effects appearing in some but not other groups, all of which had substantial numbers of city dwellers. (*b*) The size of urban-rural differentials varies by site. Where large differentials exist, such as those for esophagus, lung, and bronchus, adjustments for residence could be potentially important, but one would not anticipate striking changes for sites with more modest differentials. (*c*) Wherever the national data indicated below-average risks, reference to the metropolitan areas will often depress the estimated SMR values and emphasize the divergence of the groups in question from the over-all metropolitan experience. (*d*) Not all ethnic groups were well represented in each Standard Metropolitan Area, and the exceptions to complete coverage are listed in the footnotes.

Inspection of table 4 suggests that most departures from normal risk indicated in the national data for buccal cavity and pharynx, breast, uterus, ovary, prostate, bladder, leukemia, and the lymphomas carried over substantially unchanged into the metropolitan area results and no further comments on these sites are called for here.

For esophagus, the application of a different yardstick reduced the estimates of excess risk among all ethnic groups, several of them to the point where they did not depart markedly from the total experience of metropolitan whites. The following groups survived this test and still exhibited high SMR's: Ireland, Czechoslovakia, Poland (males), and USSR (females). One note of caution is needed concerning this set of comparisons. Widespread excess rates among the foreign-born and their im-

TABLE 4.—Standardized cancer-mortality ratios* (SMR = 100 for all whites in Standard Metropolitan Areas) with number of deaths observed for foreign-born whites, by country of birth for selected primary sites, by sex, six Standard Metropolitan Areas†, 1950

Site (International List Numbers, sixth revision, 1948)	England and Wales	Ireland	Norway	Sweden	Germany	Austria	Czechoslovakia	Poland	USSR	Italy	Canada	Mexico
MALES												
All sites (140–205)												
Observed	499	624	126	289	1118	594	317	1372	1470	1768	336	95
SMR	101	112	99	97	103	102	114	121	106	89	111	91
Buccal cavity and pharynx (140–148)												
Observed	26	49	11	5	41	11	13	48	11	76	21	2
SMR	133	220	220	42	94	47	115	102	20	95	165	51
Digestive system (150–159)												
Observed	230	335	67	155	580	293	175	665	736	869	140	40
SMR	103	130	114	112	116	109	134	128	116	96	106	96
Esophagus (150)												
Observed	16	41	5	10	46	23	20	80	48	88	15	2
SMR	91	192	104	83	112	104	165	175	93	116	116	80
Stomach (151)												
Observed	65	86	34	70	198	91	69	259	257	243	44	16
SMR	90	106	184	157	125	108	163	156	129	85	103	117
Intestines (152–153)												
Observed	57	92	6	30	122	65	36	107	179	203	37	8
SMR	106	146	42	90	100	101	115	88	118	93	118	85
Rectum (154)												
Observed	41	63	10	20	92	49	29	79	100	146	22	2
SMR	122	154	109	97	119	116	151	99	100	101	115	36
Pancreas (157)												
Observed	30	35	6	15	60	33	8	81	83	90	12	6
SMR	120	125	90	99	112	113	62	143	117	89	78	105
Lung and bronchus (162–163)												
Observed	77	49	13	30	151	117	43	286	274	270	52	24
SMR	99	54	61	68	90	115	96	142	112	77	107	128
Prostate (177)												
Observed	48	60	16	35	101	35	23	71	77	103	42	5
SMR	101	124	148	114	97	77	99	85	73	68	151	63
Bladder (181)												
Observed	27	16	3	12	51	23	11	39	63	75	14	9
SMR	112	59	50	81	96	83	86	75	96	80	102	205

Leukemia (204)												
Observed	13	14	—	8	32	14	11	35	60	63	9	—
SMR	80	80	—	87	94	78	125	97	140	102	83	—
Lymphomas (200–203, 205)												
Observed	14	14	2	12	24	32	9	49	63	72	14	3
SMR	85	76	45	135	70	165	102	123	134	106	117	55
FEMALES												
All sites (140–205)												
Observed	458	646	117	237	967	464	247	880	1156	922	387	124
SMR	106	104	107	91	99	101	101	105	116	78	107	119
Buccal cavity and pharynx (140–148)												
Observed	2	8	—	4	7	4	2	7	5	7	8	1
SMR	44	140	—	148	69	100	87	92	57	63	216	111
Digestive system (150–159)												
Observed	206	322	56	99	438	212	120	405	540	424	151	51
SMR	116	121	123	87	104	112	119	122	133	88	108	148
Esophagus (150)												
Observed	7	9	1	1	13	1	4	8	35	5	5	1
SMR	152	120	83	33	111	19	148	92	318	38	152	167
Stomach (151)												
Observed	61	98	24	35	138	54	53	124	133	112	41	13
SMR	138	146	212	121	129	116	212	152	133	95	118	160
Intestines (152–153)												
Observed	67	103	11	25	139	72	26	104	179	119	51	7
SMR	111	115	72	66	98	114	77	93	131	73	108	58
Rectum (154)												
Observed	22	40	3	14	48	27	13	59	57	50	22	5
SMR	91	109	50	91	84	103	97	128	101	74	120	109
Pancreas (157)												
Observed	21	38	8	8	39	29	9	39	55	52	14	10
SMR	117	142	174	68	92	151	88	114	132	106	99	244
Lung and bronchus (162–163)												
Observed	21	21	7	8	33	21	8	35	67	31	13	9
SMR	136	95	179	85	94	126	90	114	184	73	102	250
Breast (170)												
Observed	79	121	26	39	193	78	36	125	155	144	87	11
SMR	97	104	126	84	107	87	78	75	79	62	123	48
Cervix (171)												
Observed	29	28	6	15	46	19	13	53	27	51	22	15
SMR	123	97	115	128	99	81	107	114	52	85	94	188

See footnotes at end of table.

TABLE 4.—Standardized cancer-mortality ratios* (SMR = 100 for all whites in Standard Metropolitan Areas) with number of deaths observed for foreign-born whites, by country of birth for selected primary sites, by sex, six Standard Metropolitan Areas†, 1950—Continued

Site (International List Numbers, sixth revision, 1948)	England and Wales	Ireland	Norway	Sweden	Germany	Austria	Czechoslovakia	Poland	USSR	Italy	Canada	Mexico
FEMALES—Continued												
Uterus, other and unspecified (172–173)												
Observed	26	30	1	12	40	17	13	53	36	60	20	7
SMR	108	91	15	75	75	66	82	104	65	93	100	113
Ovary (175)												
Observed	19	28	7	11	52	32	9	43	76	44	17	5
SMR	78	83	115	86	102	115	66	83	124	61	81	68
Bladder (181)												
Observed	11	7	2	2	25	5	1	5	16	11	7	3
SMR	106	47	80	26	104	53	18	30	79	46	91	150
Leukemia (204)												
Observed	9	13	4	9	23	8	5	31	50	24	14	1
SMR	79	81	143	136	90	66	78	138	190	77	141	31
Lymphomas (200–203, 205)												
Observed	13	12	1	7	22	19	8	37	48	27	9	2
SMR	108	74	36	111	88	146	116	150	171	80	85	59

* The standardized mortality ratios are expressed, after adjustment for age by the indirect method, in terms of the corresponding cancer-death rates for all whites (native- and foreign-born) in each of the six Standard Metropolitan Areas; the SMR's are based on the summation of observed and expected numbers of deaths for each area.

† Six Standard Metropolitan Areas: New York-northeastern New Jersey, Chicago, Cleveland, Detroit, Los Angeles, and San Francisco-Oakland.

TABLE 5.—Standardized cancer mortality*, † (SMR=100 for native-born whites) with number of deaths observed for foreign-born whites by country of birth for selected primary sites, by sex, for six Standard Metropolitan Areas‡ and Regional Population outside the Standard Metropolitan Areas, Northeast, East North Central, and Western Regions, 1950

Site (International List Numbers, sixth revision, 1948)	England and Wales	Ireland	Norway§	Sweden‖	Germany	Austria¶	Czechoslovakia**	Poland††	USSR	Italy	Canada‡‡	Mexico§§
MALES												
All sites (140–205)												
Observed												
Standard Metropolitan Areas	499	623	126	289	1118	594	318	1372	1470	1768	336	95
Outside Standard Metro. Areas	456	219	215	352	850	253	211	634	317	739	504	104
SMR												
Standard Metropolitan Areas	122	133	120	121	125	122	148	151	126	106	134	107
Outside Standard Metro. Areas	105	121	108	103	115	106	114	130	132	94	127	78
Buccal cavity and pharynx (140–148)												
Observed												
Standard Metropolitan Areas	26	49	11	5	41	11	13	48	11	76	21	2
Outside Standard Metro. Areas	15	12	7	7	22	12	10	22	9	37	23	1
SMR												
Standard Metropolitan Areas	158	250	256	54	112	53	151	128	22	107	221	61
Outside Standard Metro. Areas	87	164	93	53	76	124	137	109	91	113	148	19
Digestive system (150–159)												
Observed												
Standard Metropolitan Areas	230	335	67	155	580	293	175	665	736	869	140	40
Outside Standard Metro. Areas	231	99	99	180	441	126	114	313	154	378	233	60
SMR												
Standard Metropolitan Areas	133	170	152	153	153	143	190	174	151	125	135	118
Outside Standard Metro. Areas	125	129	118	123	138	123	143	150	153	115	140	118
Esophagus (150)												
Observed												
Standard Metropolitan Areas	16	41	5	10	46	23	20	80	48	88	15	2
Outside Standard Metro. Areas	12	8	8	13	30	13	14	48	9	34	10	4

See footnotes at end of table.

TABLE 5.—Standardized cancer mortality*, † (SMR=100 for native-born whites) with number of deaths observed for foreign-born whites by country of birth for selected primary sites, by sex, for six Standard Metropolitan Areas‡ and Regional Population outside the Standard Metropolitan Areas, Northeast, East North Central, and Western Regions, 1950—Continued

Site (International List Numbers, sixth revision, 1948)	England and Wales	Ireland	Norway§	Sweden‖	Germany	Austria¶	Czechoslovakia**	Poland††	USSR	Italy	Canada‡‡	Mexico§§
MALES—Continued												
Digestive system (150–159)—Continued												
Esophagus (150)—Continued												
SMR												
Standard Metropolitan Areas	172	345	179	189	207	181	351	329	155	199	273	143
Outside Standard Metro. Areas	118	195	216	169	178	213	318	361	167	168	114	160
Stomach (151)												
Observed												
Standard Metropolitan Areas	65	86	34	70	198	91	69	259	257	243	44	16
Outside Standard Metro. Areas	93	34	50	81	186	51	45	133	65	115	91	33
SMR												
Standard Metropolitan Areas	123	150	264	223	175	155	252	234	183	121	140	147
Outside Standard Metro. Areas	161	142	180	171	183	165	188	215	208	119	171	202
Intestines (152–153)												
Observed												
Standard Metropolitan Areas	57	92	6	30	122	65	36	107	179	203	37	8
Outside Standard Metro. Areas	49	26	16	37	92	24	20	52	30	89	54	4
SMR												
Standard Metropolitan Areas	125	171	51	112	119	116	146	104	135	107	139	101
Outside Standard Metro. Areas	100	123	77	99	107	90	94	92	116	102	123	35
Rectum (154)												
Observed												
Standard Metropolitan Areas	41	63	10	20	92	49	29	79	100	146	22	2
Outside Standard Metro. Areas	23	12	11	18	58	14	10	27	13	58	30	4
SMR												
Standard Metropolitan Areas	154	194	139	132	151	144	207	130	123	125	148	44
Outside Standard Metro. Areas	80	95	96	86	126	90	85	82	84	109	120	54

Pancreas (157)												
Observed												
Standard Metropolitan Areas	30	35	6	15	60	33	8	81	83	90	12	6
Outside Standard Metro. Areas	37	11	8	12	34	11	10	20	16	38	22	6
SMR												
Standard Metropolitan Areas	143	154	113	124	137	139	75	176	144	109	94	122
Outside Standard Metro. Areas	169	125	73	66	91	89	103	83	122	94	108	77
Respiratory system (160–165)												
Observed												
Standard Metropolitan Areas	91	74	17	35	177	128	49	328	298	308	55	28
Outside Standard Metro. Areas	68	32	10	29	94	40	40	134	67	122	68	15
SMR												
Standard Metropolitan Areas	134	89	89	95	119	139	136	190	132	95	135	167
Outside Standard Metro. Areas	106	120	36	61	99	100	134	157	161	82	117	61
Lung and bronchus (162–163)												
Observed												
Standard Metropolitan Areas	77	49	13	30	151	117	43	286	274	270	52	24
Outside Standard Metro. Areas	60	24	9	27	77	34	33	120	65	97	56	15
SMR												
Standard Metropolitan Areas	132	69	81	95	119	147	139	192	140	97	148	161
Outside Standard Metro. Areas	111	107	36	66	97	100	130	163	182	75	112	68
Prostate (177)												
Observed												
Standard Metropolitan Areas	48	60	16	35	101	35	23	71	77	103	42	5
Outside Standard Metro. Areas	61	37	38	69	114	23	15	41	19	55	74	11
SMR												
Standard Metropolitan Areas	93	111	134	109	89	69	92	79	65	60	142	63
Outside Standard Metro. Areas	97	136	130	132	96	76	64	72	67	66	131	87
Bladder (181)												
Observed												
Standard Metropolitan Areas	27	16	3	12	51	23	11	39	63	75	14	9
Outside Standard Metro. Areas	20	9	12	14	45	9	5	28	13	25	24	1
SMR												
Standard Metropolitan Areas	125	64	56	96	106	88	99	84	102	84	117	231
Outside Standard Metro. Areas	84	85	117	76	111	73	53	106	108	61	114	18

See footnotes at end of table.

TABLE 5.—Standardized cancer mortality*, † (SMR=100 for native-born whites) with number of deaths observed for foreign-born whites by country of birth for selected primary sites, by sex, for six Standard Metropolitan Areas‡ and Regional Population outside the Standard Metropolitan Areas, Northeast, East North Central, and Western Regions, 1950—Continued

Site (International List Numbers, sixth revision, 1948)	England and Wales	Ireland	Norway§	Sweden‖	Germany	Austria¶	Czechoslovakia**	Poland††	USSR	Italy	Canada‡‡	Mexico§§
MALES—Continued												
Leukemia (204)												
Observed												
Standard Metropolitan Areas	13	14	—	8	32	14	11	35	60	63	9	—
Outside Standard Metro. Areas	10	6	13	9	25	7	7	14	11	24	19	4
SMR												
Standard Metropolitan Areas	88	89	—	96	106	90	143	107	159	116	89	—
Outside Standard Metro. Areas	67	111	178	73	99	83	109	87	120	88	128	63
Lymphomas (200–203, 205)												
Observed												
Standard Metropolitan Areas	14	14	2	12	24	32	9	49	63	72	14	3
Outside Standard Metro. Areas	10	5	6	7	26	4	6	16	11	27	14	—
SMR												
Standard Metropolitan Areas	95	85	50	152	77	190	120	141	151	120	131	59
Outside Standard Metro. Areas	72	94	90	61	119	48	94	101	122	92	99	—
All other sites												
Observed												
Standard Metropolitan Areas	50	62	10	27	112	58	26	137	162	202	41	8
Outside Standard Metro. Areas	41	19	30	37	83	32	14	66	33	71	49	12
SMR												
Standard Metropolitan Areas	100	110	78	95	103	100	98	120	116	101	123	59
Outside Standard Metro. Areas	80	93	124	90	95	115	63	114	113	74	97	59

FEMALES

All sites (140–205)												
Observed												
Standard Metropolitan Areas	458	646	117	237	967	464	247	880	1156	922	387	124
Outside Standard Metro. Areas	398	220	165	220	700	136	136	311	197	366	481	118
SMR												
Standard Metropolitan Areas	112	111	114	98	105	107	109	112	122	83	113	127
Outside Standard Metro. Areas	103	106	115	90	106	90	102	88	119	85	117	121
Buccal cavity and pharynx (140–148)												
Observed												
Standard Metropolitan Areas	2	8	—	4	7	4	2	7	5	7	8	1
Outside Standard Metro. Areas	3	7	1	3	2	3	1	1	—	3	6	—
SMR												
Standard Metropolitan Areas	51	140	—	167	79	100	100	100	56	64	286	167
Outside Standard Metro. Areas	81	333	71	125	30	176	67	26	—	71	140	—
Digestive system (150–159)												
Observed												
Standard Metropolitan Areas	206	322	56	99	438	212	120	405	540	424	151	51
Outside Standard Metro. Areas	157	102	73	103	351	58	68	132	95	164	192	48
SMR												
Standard Metropolitan Areas	132	142	144	102	121	133	141	144	157	104	121	164
Outside Standard Metro. Areas	102	119	129	102	128	102	137	99	157	104	122	156
Esophagus (150)												
Observed												
Standard Metropolitan Areas	7	9	1	1	13	1	4	8	35	5	5	1
Outside Standard Metro. Areas	7	5	3	4	8	1	1	3	4	3	6	—
SMR												
Standard Metropolitan Areas	226	196	167	59	171	34	222	145	473	56	200	250
Outside Standard Metro. Areas	219	217	273	182	143	63	143	107	267	97	207	—
Stomach (151)												
Observed												
Standard Metropolitan Areas	61	98	24	35	138	54	53	124	133	112	41	13
Outside Standard Metro. Areas	48	35	28	31	122	22	22	42	30	55	46	18
SMR												
Standard Metropolitan Areas	191	213	304	167	184	178	314	233	202	143	156	200
Outside Standard Metro. Areas	144	197	219	137	201	191	227	160	240	179	137	265
Intestines (152–153)												
Observed												
Standard Metropolitan Areas	67	103	11	25	139	72	26	104	179	119	51	7
Outside Standard Metro. Areas	51	29	13	23	103	17	16	33	25	35	75	6

See footnotes at end of table.

TABLE 5.—Standardized cancer mortality*, † (SMR=100 for native-born whites) with number of deaths observed for foreign-born whites by country of birth for selected primary sites, by sex, for six Standard Metropolitan Areas‡ and Regional Population outside the Standard Metropolitan Areas, Northeast, East North Central, and Western Regions, 1950—Continued

Site (International List Numbers, sixth revision, 1948)	England and Wales	Ireland	Norway§	Sweden‖	Germany	Austria¶	Czechoslovakia**	Poland††	USSR	Italy	Canada‡‡	Mexico§§
FEMALES—Continued												
Digestive system (150–159)—Continued												
SMR												
Standard Metropolitan Areas	115	116	74	69	100	114	80	95	131	74	112	64
Outside Standard Metro. Areas	88	87	65	64	102	80	88	64	112	58	128	55
Rectum (154)												
Observed												
Standard Metropolitan Areas	22	40	3	14	48	27	13	59	57	50	22	5
Outside Standard Metro. Areas	16	16	10	7	37	4	8	12	11	22	22	7
SMR												
Standard Metropolitan Areas	103	124	59	109	96	118	113	149	114	85	135	119
Outside Standard Metro. Areas	77	138	135	54	106	53	125	66	134	97	102	167
Pancreas (157)												
Observed												
Standard Metropolitan Areas	21	38	8	8	39	29	9	39	55	52	14	10
Outside Standard Metro. Areas	12	7	7	18	25	6	9	18	12	9	20	3
SMR												
Standard Metropolitan Areas	134	175	211	81	112	185	108	141	164	131	109	263
Outside Standard Metro. Areas	74	81	113	171	91	105	176	135	185	57	124	86
Respiratory system (160–165)												
Observed												
Standard Metropolitan Areas	24	22	8	10	35	22	9	37	73	37	14	9
Outside Standard Metro. Areas	23	10	8	10	31	2	5	20	12	19	5	10
SMR												
Standard Metropolitan Areas	167	109	229	115	109	150	114	140	229	99	113	243
Outside Standard Metro. Areas	163	135	148	109	129	39	109	167	200	127	34	278
Lung and bronchus (162–163)												
Observed												
Standard Metropolitan Areas	21	21	7	8	33	21	8	35	67	31	13	9
Outside Standard Metro. Areas	19	9	7	7	28	2	4	16	12	17	5	10
SMR												
Standard Metropolitan Areas	165	122	241	108	118	165	118	148	241	95	124	281
Outside Standard Metro. Areas	162	138	140	89	137	42	93	155	231	131	39	333
Breast (170)												
Observed												
Standard Metropolitan Areas	79	121	26	39	193	78	36	125	155	144	87	11

Breast (170)												
Observed												
Standard Metropolitan Areas	79	121	26	39	193	78	36	125	155	144	87	11
Outside Standard Metro. Areas	81	31	18	35	102	13	22	34	26	47	86	9
SMR												
Standard Metropolitan Areas	96	100	124	85	105	84	79	75	76	60	127	53
Outside Standard Metro. Areas	110	78	69	79	85	43	84	48	78	51	107	43
Cervix (171)												
Observed												
Standard Metropolitan Areas	29	28	6	15	46	19	13	53	27	51	22	15
Outside Standard Metro. Areas	23	4	8	3	41	10	9	18	6	22	35	25
SMR												
Standard Metropolitan Areas	120	86	102	117	92	72	93	104	47	75	96	190
Outside Standard Metro. Areas	112	40	101	24	124	111	111	89	56	81	144	309
Uterus, other and unspecified (172–173)												
Observed												
Standard Metropolitan Areas	26	30	1	12	40	17	13	53	36	60	20	7
Outside Standard Metro. Areas	25	13	18	17	35	9	9	18	8	28	30	6
SMR												
Standard Metropolitan Areas	93	75	14	70	63	54	76	90	54	76	84	113
Outside Standard Metro. Areas	94	92	182	100	74	80	89	68	67	91	106	97
Ovary (175)												
Observed												
Standard Metropolitan Areas	19	28	7	11	52	32	9	43	76	44	17	5
Outside Standard Metro. Areas	19	8	10	9	23	10	4	15	15	20	29	6
SMR												
Standard Metropolitan Areas	81	85	119	90	104	118	67	86	127	62	86	72
Outside Standard Metro. Areas	96	79	135	75	75	111	53	71	152	73	128	90
Bladder (181)												
Observed												
Standard Metropolitan Areas	11	7	2	2	25	5	1	5	16	11	7	3
Outside Standard Metro. Areas	6	8	1	3	12	4	—	7	—	7	11	—
SMR												
Standard Metropolitan Areas	104	45	83	29	100	47	18	28	69	41	86	158
Outside Standard Metro. Areas	54	129	23	42	61	108	—	75	—	68	98	—
Leukemia (204)												
Observed												
Standard Metropolitan Areas	9	13	4	9	23	8	5	31	50	24	14	1
Outside Standard Metro. Areas	9	4	3	7	14	1	3	14	3	10	14	2
SMR												
Standard Metropolitan Areas	82	88	148	148	100	73	89	156	208	85	147	36
Outside Standard Metro. Areas	88	75	86	113	83	26	88	156	65	87	123	61

See footnotes at end of table.

TABLE 5.—Standardized cancer mortality*, † (SMR=100 for native-born whites) with number of deaths observed for foreign-born whites by country of birth for selected primary sites, by sex, for six Standard Metropolitan Areas‡ and Regional Population outside the Standard Metropolitan Areas, Northeast, East North Central, and Western Regions, 1950—Continued

Site (International List Numbers, sixth revision, 1948)	England and Wales	Ireland	Norway§	Sweden‖	Germany	Austria¶	Czechoslovakia**	Poland††	USSR	Italy	Canada‡‡	Mexico§§
FEMALES—Continued												
Lymphomas (200–203, 205)												
Observed												
Standard Metropolitan Areas	13	12	1	7	22	19	8	37	48	27	9	2
Outside Standard Metro. Areas	12	5	7	4	20	6	3	12	5	13	21	4
SMR												
Standard Metropolitan Areas	126	87	40	127	103	171	136	171	196	93	98	67
Outside Standard Metro. Areas	129	111	184	66	132	143	81	132	111	111	189	133
All other sites												
Observed												
Standard Metropolitan Areas	40	55	6	29	86	48	31	84	130	93	38	19
Outside Standard Metro. Areas	40	28	18	26	69	20	12	40	27	33	52	8
SMR												
Standard Metropolitan Areas	88	90	54	107	87	105	125	98	130	79	96	161
Outside Standard Metro. Areas	92	128	106	92	92	117	78	104	148	69	111	68

*The standardized mortality ratios are expressed, after adjustment for age by the indirect method, in terms of the corresponding cancer death rates for native-born whites; the SMR's are based on the summation of observed and expected numbers of deaths in each region and are thus adjusted for the regional distributions of the foreign-born groups.

†For regional grouping of States, *see* text-figure 1.

‡Six Standard Metropolitan Areas: New York-northeastern New Jersey, Chicago, Cleveland, Detroit, Los Angeles, and San Francisco-Oakland.

§Standard Metropolitan Areas include only New York-northeastern New Jersey, Chicago, and Los Angeles.

‖Standard Metropolitan Areas include only New York-northeastern New Jersey, Chicago, Los Angeles, and San Francisco-Oakland.

¶Standard Metropolitan Areas include only New York-northeastern New Jersey, Chicago, Cleveland, Detroit, and Los Angeles.

**Standard Metropolitan Areas include only New York-northeastern New Jersey, Chicago, and Cleveland.

††Standard Metropolitan Areas include only New York-northeastern New Jersey, Chicago, Cleveland, Detroit, and Los Angeles.

‡‡Standard Metropolitan Areas include only New York-northeastern New Jersey, Chicago, Detroit, Los Angeles, and San Francisco-Oakland.

§§Standard Metropolitan Areas include only Chicago, Los Angeles, and San Francisco-Oakland.

mediate descendants could have resulted in an unusually high level of esophagus rates in metropolitan areas, so that under these conditions an SMR value close to 100 should not be taken as positive evidence against an excess risk.

The previous comments for esophagus apply to stomach as well. Ethnic groups for which high SMR values persisted for both sexes in the metropolitan areas included Norway, Sweden, Czechoslovakia, Poland, and possibly USSR. The excess risk among Italian migrants in the national data was converted into a slight deficit when measured against the experience of metropolitan whites. While the magnitude of the SMR's was diminished for intestines and rectum in the metropolitan series, the most prominent feature—an excess risk for Irish males—remained substantially unchanged.

The lung was another site in which the choice of a different standard reduced the scale of excess risks. The high SMR's for Polish males and Russian females still stood out, however. The presentation did emphasize the low rate for Italian males as compared to the rates for other groups in the metropolitan areas.

Table 5 demonstrates that most of the important ethnic-group deviations from the native-white experience shown in the national data, particularly those significant at the 1 percent level, remained apparent both inside and outside the largest metropolitan centers, so that the ethnic-group effects did not rise solely from conditions associated with these conurbations. It should be remembered that the outlying areas covered smaller urban communities and rural populations, so that the probable magnitude of ethnic-group effects found in strictly rural settings is a matter for further study. While there were no noteworthy exceptions to the general concordance of SMR's within and without the Standard Metropolitan Areas, particularly when the number of observations and the potential sampling errors are taken into account, the suggestion of higher metropolitan male risks for the intestines and rectum (Ireland) and for the lung (England and Poland) should not be ignored completely.

Other Foreign-Born Groups

The absence of information on age composition precluded the computation of SMR's for the less numerous foreign-born groups. However, a judgment as to whether any of them had experienced high or low risks for certain sites may be hazarded from the proportionate distribution by site of deaths from cancer in country of birth. There are some uncertainties in this "relative frequency" approach. The result for any one site will depend in part on the excess or deficit of deaths for other sites. Since the pattern of age-specific rates varies by site, an unusual age distribution in the population at risk may raise or depress the proportion recorded for any given site. Also, the total number of cancer deaths available for study in these groups was small and the sampling variation of the proportions sizable, so that only very striking differences in risk may be detected with any confidence.

TABLE 6.—Foreign-born groups with unusually high or low percentage of total cancer deaths recorded for individual sites, by sex, United States (35 States), 1950*

Countries	Males		Females
	Buccal cavity and pharynx		
Ireland	*6.9%* (*224*)		
Scotland	5.5%		
Czechoslovakia	*4.1%* (*138*)		
Finland	1.7%		
Lithuania	1.6%		
Mexico	*1.4%* (*34*)		
USSR	*1.3%* (*39*)		
	Esophagus		
Lithuania	7.7%	*USSR*	*2.8%* (*442*)
Hungary	6.8%	Lithuania	2.5%
Poland	*6.2%* (*336*)	Finland	2.3%
		Czechoslovakia	*1.4%* (*222*)
Mexico	*2.8%* (*146*)		
Denmark	1.0%		
Greece	0.8%		
	Stomach		
Finland	32.1%		
Denmark	27.0%		
Norway	*23.5%* (*189*)		
Poland	*19.4%* (*225*)		
	Intestines		
Finland	6.2%	Denmark	18.4%
Switzerland	6.0%	*USSR*	*14.8%* (*128*)
Mexico	*5.5%* (*58*)		
Greece	4.4%		
	Rectum		
Belgium	12.0%		
Ireland	*8.8%* (*174*)		
Sweden	*4.7%* (*86*)		
Finland	3.7%		
Mexico	*2.8%* (*48*)		
	Lung and bronchus		
Greece	26.5%	*Mexico*	*7.9%* (*313*)
Yugoslavia	23.4%	*USSR*	*6.1%* (*249*)
Scotland	22.1%	Romania	6.0%
Poland	*20.4%* (*188*)		
		Uterus	
		USSR	*5.8%* (*54*)
		Finland	5.2%
		Romania	4.5%

*SMR's for major foreign-born groups shown in parentheses.

Table 6 summarizes some of the more suggestive relative frequency results for foreign-born groups, most of them relating to observations on males. For orientation and as an aid in interpretation, the corresponding percentages for major ethnic groups with high or low SMR's for the various sites have been included. No extreme outliers in the relative proportions for the breast, ovary, bladder, leukemia, and lymphomas were noted among the minor foreign-born groups. While no great weight should be attached to any single entry in table 6, the data were consistent with other sources of information on many points.

Reports on cancer morbidity and mortality from the countries of origin lend credence to several inferences drawn from the relative frequency data. The high proportion of deaths for the buccal cavity and the lung among males migrating from Scotland had its counterpart in mortality rates in Scotland (*24*, *25*); the rates for the lung were among the highest recorded anywhere. The excess of stomach-cancer deaths among persons from Finland and the deficits for buccal cavity, intestines and rectum, and uterus conformed to the prevailing experience in that country (*25*, *32*). The low sex ratio for esophagus characteristic of Finland and due to the elevated rate for females (*31*, *32*) carried over into the U.S. data for this group.

The contrast in the relative frequency ranking for esophagus (low) and stomach (high) among Danish males in the United States corresponded with the situation in the mother country (*25*). Mortality from cancer of the esophagus in Denmark was low in comparison to Norway and Sweden, while Danish mortality for stomach cancer was in line with the high death rates elsewhere in Scandinavia.

Since Belgium ranked high in international comparisons of mortality from cancer of the rectum, the concentration of deaths from cancer in this site among Belgian males in the United States is worth some attention.

No mortality data are available for Greece and Yugoslavia as a basis for discussion of the excessive frequency of lung-cancer deaths among males from these countries in the United States. The present findings were consistent with the excess lung-cancer risks among these two ethnic groups in Ohio reported by Mancuso (*11*).

The relative frequency data also indicated that the risks reported for certain sites among immigrants from Poland and USSR were not confined to those groups, but might have prevailed among persons from some bordering states (Finland, Hungary, Lithuania, and Romania). The close correspondence between Romania and the USSR, with regard to a high risk for lung and bronchus and a low risk for uterus, was interesting since Jews constituted a sizable bloc of the migrants from these two countries.

No mortality or incidence data are generally available for East European countries, with the exception of Finland. For this reason, the American information on ethnic groups may provide some of the better clues as to the true distribution of cancer risk by site among the populations of those countries.

Comparisons With Countries of Origin

We turn now to direct contrasts of the experience of migrant groups with mortality prevailing in the countries of origin. The underlying idea is not new and was attempted by Lombard and Doering (*8*) over 30 years ago with the rudimentary data from foreign countries then at their disposal. Present information now affords better opportunities for comparisons. Standards of diagnosis and death certifications have gradually improved and the national offices of vital statistics now pay greater at-

TABLE 7.—Standardized cancer-mortality ratios* (SMR=100 for residents in country of origin) with number of deaths for foreign-born whites by country of birth for selected primary sites, by sex, United States (35 States), 1950

Site (International List Numbers, sixth revision, 1948)	England and Wales	Ireland	Norway	Sweden	Germany	Italy	Canada
MALES							
All sites (140–205)							
Observed	1114	928	515	899	2428	2671	1052
SMR	92	148	110	110	99	119	112
Buccal cavity and pharynx (140–148)							
Observed	45	64	24	19	75	122	52
SMR	99	136	209	133	270	124	143
Digestive system (150–159)							
Observed	545	473	249	458	1265	1335	482
SMR	95	136	90	100	83	99	101
Esophagus (150)							
Observed	32	53	22	29	90	126	30
SMR	75	237	162	144	114	155	120
Stomach (151)							
Observed	190	131	121	210	491	388	167
SMR	86	87	69	88	63	50	85
Lung and bronchus (152–153)							
Observed	160	(not	37	83	264	382	130
SMR	54	available)	159	160	90	169	112
Prostate (177)							
Observed	128	113	81	145	286	173	144
SMR	125	230	121	127	132	145	132
FEMALES							
All sites (140–205)							
Observed	945	925	392	663	1983	1384	1095
SMR	109	135	111	101	87	110	107
Buccal cavity and pharynx (140–148)							
Observed	5	15	2	11	9	10	15
SMR	39	87	50	98	79	96	134
Digestive system (150–159)							
Observed	403	455	182	307	951	632	438
SMR	98	125	104	93	70	98	96
Esophagus (150)							
Observed	15	15	4	10	25	9	13
SMR	77	64	88	85	81	74	100
Stomach (151)							
Observed	125	140	75	107	315	176	107
SMR	87	100	75	66	52	55	80
Lung and bronchus (152–153)							
Observed	41	(not	19	22	73	51	26
SMR	90	available)	214	100	125	130	86
Breast (170)							
Observed	178	162	63	105	336	206	210
SMR	110	148	140	117	153	118	113
Uterus (171–174)							
Observed	115	(not	39	64	191	175	136
SMR	148	available)	156	142	106	100	121
Ovary (175)							
Observed	42	39	21	31	(not	65	60
SMR	90	200	103	95	available)	266	120

* The standardized mortality ratios are expressed after adjustment for age by the indirect method in terms of contemporary cancer-death rates recorded in the country of origin.

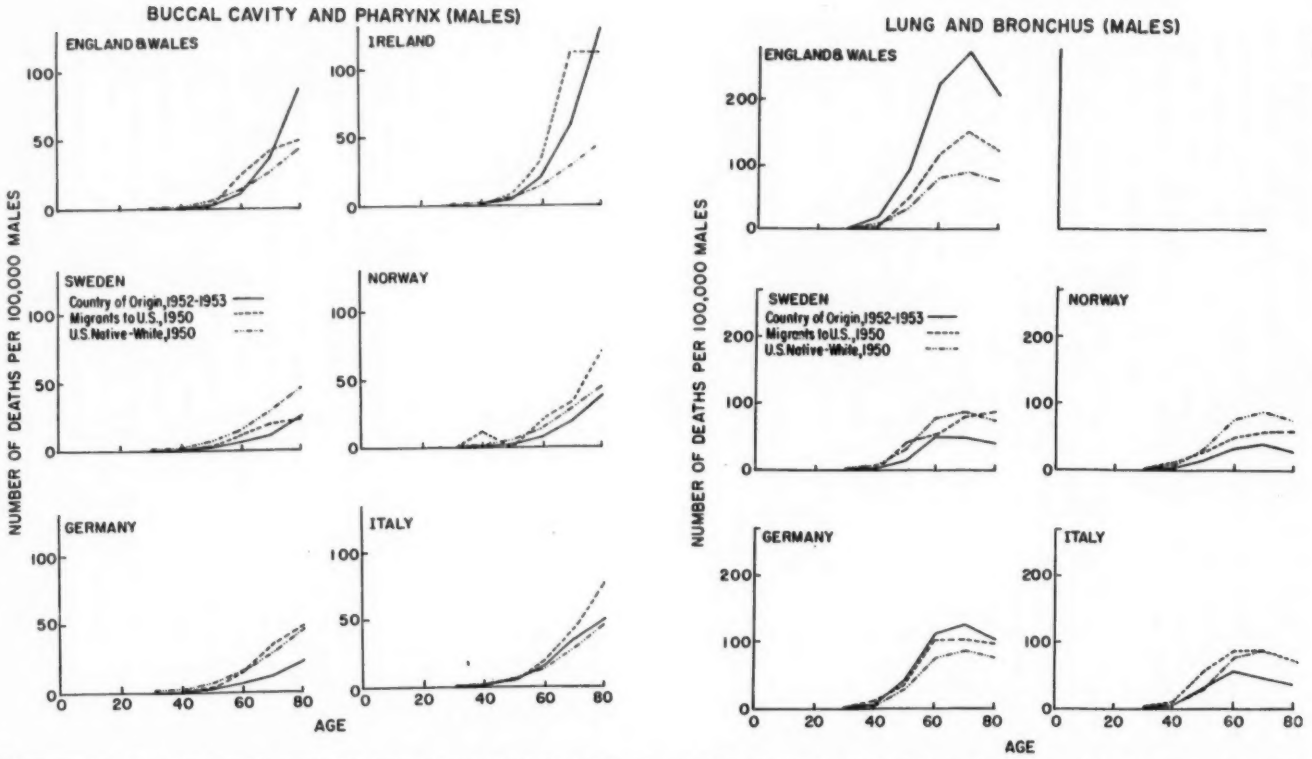
tention to site detail in reporting on cancer deaths. Segi (*25*) and Case and Harley (*24*) have systematically assembled age-specific death rates for a limited number of sites from 23 countries, 7 of which are represented by sizable migrant groups in the United States. Their materials have been used to compute the standardized mortality ratios given in table 7.

It should be emphasized that these comparisons and the ones in text-figures 7 to 10 are intended only as rough first approximations, with full awareness of the difficulties introduced by lack of uniformity among countries in diagnostic concepts and rules for coding causes of death. Selective migration factors pose additional complications. Since migrants were presumably subject to selection by health status, socioeconomic class, locality, etc., there is no assurance that comprehensive national data from countries of origin would be truly comparable with those for their migrants to the United States. Also, for some sites with strong urban-rural differentials, comparisons of predominantly urban groups of migrants with composite urban-rural groups in the home country would be imprecise. Nevertheless, a start must be made somewhere and with these qualifications in mind, inspection of the data for suggestions of broad patterns may prove instructive.

For all sites combined, the elevated risk among Irish migrants of both sexes was noteworthy. The departures from the level of cancer mortality of the six other migrant groups in the home countries were not nearly as great.

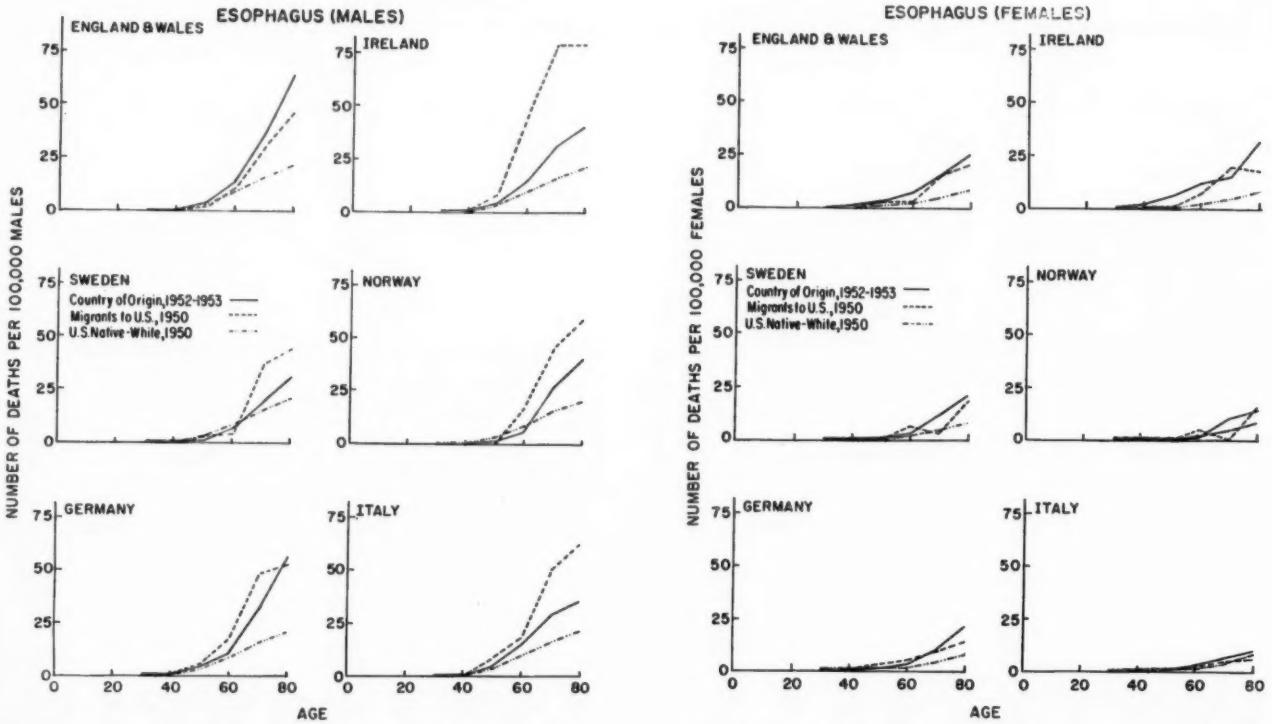
While the SMR's for cancer of the buccal cavity and pharynx were still high, the experience of Irish migrants more closely resembled that of persons in Ireland than of U.S. native whites. All migrant males except those from England exhibited buccal-cancer rates higher than for the country of origin, a relationship which did not hold true for females. Although the number of deaths for Swedish females was small, there was no indication of a marked change from the high buccal-cancer rate prevailing among that sex in Sweden. The apparent excess for esophageal cancer among male migrants was reduced when the country of origin was introduced as the standard for comparison. Perhaps the most intriguing aspect of the results observed for esophagus was that female migrants experienced risks lower than for the country of origin; the sex differential in the pattern of the SMR's for this site stood out clearly.

While the stomach-cancer rates for migrants of both sexes ranged below those in the countries of origin, more emphasis might perhaps be placed on the similarities rather than the discrepancies. For total digestive system, the correspondence between migrants and parent populations was close, except for the Irish; this raises the possibility that some of the apparent differences for stomach cancer in favor of the migrants may merely reflect overcertification of this site at the expense of other sites in the digestive system, in the other countries concerned. Even for Italian migrants with stomach-cancer SMR's slightly over 50, this interpretation cannot be completely ruled out in view of digestive-system SMR's very close to 100. The digestive-system SMR's for Irish migrants, which were

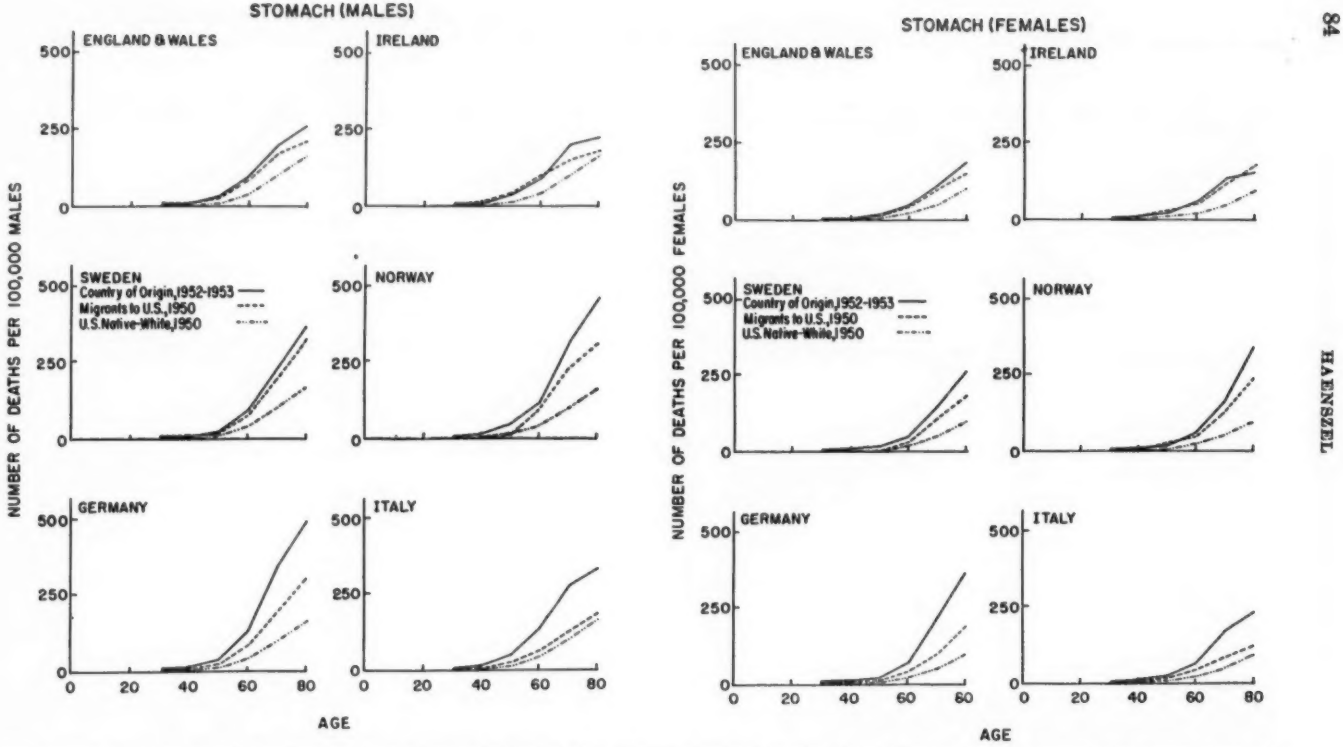


TEXT-FIGURE 7.—Age-specific mortality from cancer of selected sites among migrants to the United States from 6 countries in comparison with U.S. native whites and populations in countries of origin.

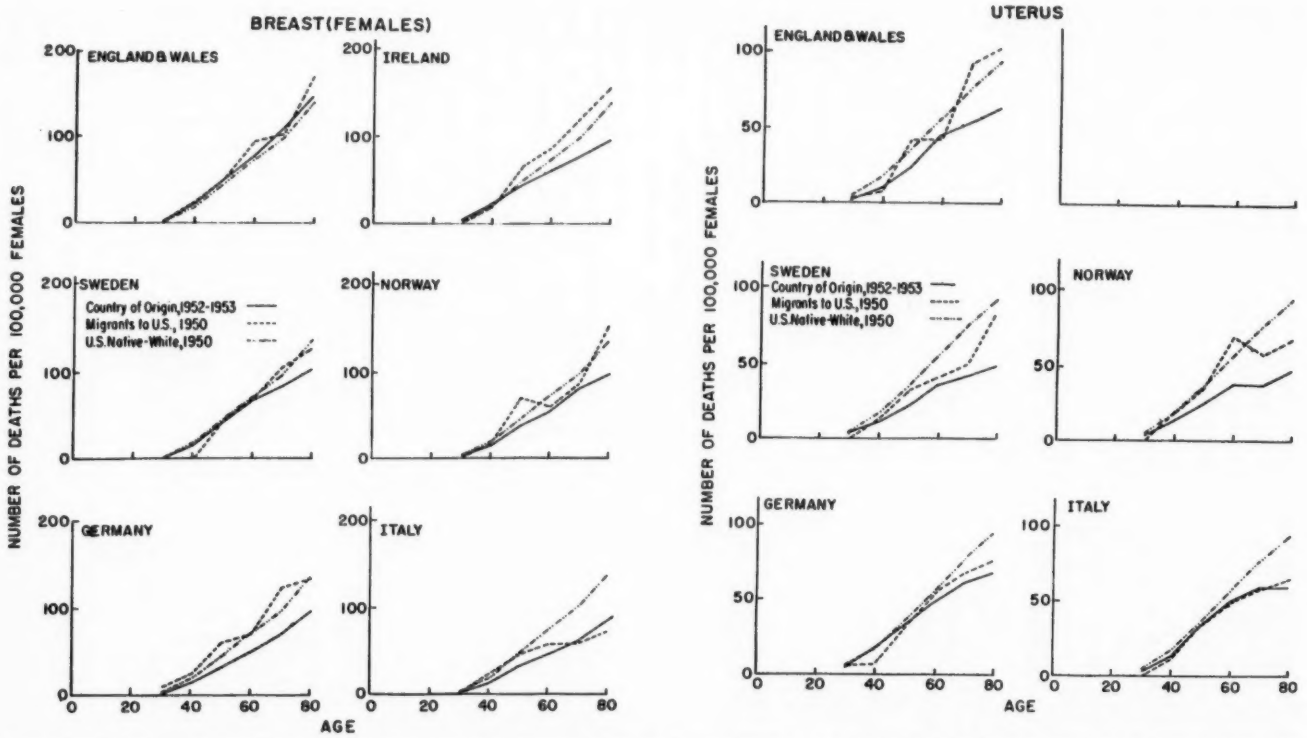
U.S. native whites and populations in countries of origin.



TEXT-FIGURE 8.—Age-specific mortality from cancer of selected sites among migrants to the United States from 6 countries in comparison with U.S. native whites and populations in countries of origin.



TEXT-FIGURE 9.—Age-specific mortality from cancer of selected sites among migrants to the United States from 6 countries in comparison with U.S. native whites and populations in countries of origin.



TEXT-FIGURE 10.—Age-specific mortality from cancer of selected sites among migrants to the United States from 6 countries in comparison with U.S. native whites and populations in countries of origin.

well above 100, represented the contributions of excess risks for esophagus, intestines, and rectum similar in nature to those found in the contrasts with native whites. Irrespective of the standard of comparison adopted, high digestive-system risks persist as a prominent characteristic of this latter group.

The SMR's for lung and bronchus based on experience abroad presented a variable picture. Males from England, and possibly Germany, exhibited lower rates, while those from Norway, Sweden, and Italy were subjected by virtue of their change of residence to higher risks. These seeming inconsistencies are resolved in text-figure 7 which makes plain the general principle of a partial displacement in the lung and bronchus rates for migrants from the level prevailing in the home country toward the rate predominant in the host country. While there were some points of similarity between the two sexes, the relationships for the female rates were less clear-cut.

All seven groups of females, particularly those from Germany, Ireland, and Norway, experienced higher breast-cancer rates than women remaining in the countries of origin, so that the relatively high ranking of the United States in international comparisons covered migrants from lower-risk countries as well. Thus, despite the favorable experience of Italian females *vis-à-vis* native whites, migration from Italy to the United States can scarcely be claimed to confer a protective effect. The rather close correspondence between migrants and residents of Italy was interesting in another connection. The persistently low rates reported for breast cancer, an accessible site and a disease relatively easy to diagnose, have caused the Italian vital statistics to become suspect and have led students in other countries to place little confidence in them. These U.S. data, however, would support the presence of a truly low risk for breast cancer in Italy as against failure to gather accurate mortality data. A comment along similar lines is stimulated by the rather large urban-rural differential noted for this site in Norway, where the experience of the urban population resembles that observed elsewhere so that the unique feature of the international pattern is the lower risk in rural Norway (*48*). The question arises whether the elevated SMR for Norwegian migrants shown in table 7, which represents a risk about equal to that for U.S. native whites, may reflect the results of removal of women from a rural environment which, in a manner not now understood. conferred a protective effect.

The uterine cancer risks for women from England, Norway, and Sweden were well above those reported in the home countries, although their rates did not constitute unusual departures from the U.S. native-white standard. The favorable position of Italian migrants with respect to other American groups seemed to result from a continuation of the same level of risk noted in Italy.

Ovary and prostate cancer resembled each other in pinpointing the same two migrant groups—Irish and Italians—as exhibiting very sizable increases in risk when compared to the home countries. To what extent this could be an artifact due to diagnostic and related factors is conjectural.

To place in perspective the experience of migrants as related to the situation in home and host countries, we have given in text-figures 7 to 10 the age-specific rates for 6 sites for 6 migrant groups. Canadians were excluded because of the trivial nature of the differences in rates between the United States and Canada. Only the male data were shown for buccal cavity and lung, since the small number of deaths among female migrants yielded unstable age-specific rates. In fact, no great weight should be attached to any single age-specific rate for migrants because of the potentially large sampling errors. The patterns of the specific rates, however, generally appear to support inferences to be drawn from the summary age-adjusted SMR's.

Some generalizations may be ventured on a type classification of the relationships exhibited for the various sites, even though exceptions can be recognized. For esophagus (males) and to a lesser degree for buccal cavity (males) the migrant rates tended to be higher than those in the home country or among native whites in this country. (Female esophagus did not conform to the male pattern.) The stomach rates for migrants were aligned more closely to the country of origin; even the Italian experience might be reconciled to this pattern, if overcertification of this site could be demonstrated and the rates in Italy scaled down to compensate for this. For lung and bronchus, the migrant rates tended to occupy an intermediate position, irrespective of whether the rates for country of origin were higher or lower than those for the United States. The several groups presented mixed patterns for breast and uterus which defied simple categorization.

The higher migrant rates for male esophagus, in particular, may reflect as suggested earlier, the role of urban-linked factors. If the comparisons for esophagus could be adjusted to control for the proportion of urban residents, it is conceivable that the differential between migrants and country of origin might be diminished and converted to a pattern more nearly resembling that for stomach. In view of the displacement of death rates in both directions for lung cancer among migrant groups, it seems doubtful whether control for urban residence would alter the fundamental relationships that are apparent for this site.

One impression which may remain with the reader is the contrast in the results revealed by the data for stomach and lung, and this may lead to further speculation on the nature of the underlying etiologic factors for these sites.

DISCUSSION

Examination of the American mortality data leads to the following conclusions:

(*a*) The foreign-born in the United States have been subject to different schedules of cancer risks than the native-white population. The data assembled over the past half century have consistently pointed in the same direction. Moreover, these differences between native- and foreign-born were national in scope and not confined to any single region of the country.

(*b*) The excesses and deficits in risk were not uniformly distributed among all sites or in all ethnic groups, but were specific to certain combinations of site and ethnic group. Several of the more remarkable ethnic-group results were concentrated in the upper digestive tract—esophagus and stomach—in contrast to the pattern of smaller ethnic-group variability for intestines and rectum. Many of the differences for individual sites were so striking and consistent with findings of earlier investigations that they may be regarded as firmly established and not likely to be upset by more extensive observations or the collection of data emphasizing more strict attention to diagnostic detail. The latter statement should not be construed as suggesting that further investigation is not desirable to determine more exactly the magnitude and nature of the effects.

(*c*) While no precise statistical control of residence was possible, review of the data for Standard Metropolitan Areas indicates that concentration of foreign-born in urban areas may be ruled out as a possible explanation of the major ethnic-group effects noted.

(*d*) While not an invariable rule, there were enough similarities between the experience of migrants and their parent populations to suggest a carry-over for some sites of effects present in distant countries to persons who had long since departed. Some of the most telling evidence on this score is the persistence of characteristic sex ratios, such as those for buccal cavity among Swedish migrants and for esophagus among migrants from several countries, including Finland. Instances of transfers of low mortality (breast cancer among Italian migrants) and of high mortality (stomach cancer among Scandinavian and USSR migrants, lung cancer among English migrants) can also be cited.

Also, there were some internal consistencies in the results for migrants from related countries. When the rankings by site are scanned for four groupings—Poland, Czechoslovakia, USSR; Germany, Austria; Norway, Sweden; England, Ireland, Canada—a general correspondence in the rankings within these groups can be discerned for many sites. The background of general correspondence brings into sharper relief some of the more unusual ethnic group results which deviate from the pattern, such as the low USSR male risks for buccal cavity and esophagus, the high Swedish female risk for buccal cavity, and the high Irish male risk for esophagus.

The American ethnic-group comparisons in general support inferences on international gradients in risk for individual sites to be drawn from examination of the available European vital statistics data. Since the American data were subject to common registration and medical certification practices, they would indicate that these factors could not be responsible for all differences arising in international comparisons, but would favor instead the existence of true differences in risk.

It must be conceded that the reliability of death certificates as to diagnosis leaves something to be desired, particularly for the years immediately following 1900. However, differentials which appeared in earlier reports have persisted to the present day, concurrent with improved cause-of-death reporting, and have remained evident in the largest metropolitan areas which have major medical centers where presumably the quality of supporting diagnostic evidence would be best. A study made of a sample of death certificates filed during 1956 in Pennsylvania showed that 68 percent of the cancer certifications were supported by a microscopic examination of tissue (*49*). Certainly, reliance on mortality data for

cancer investigations poses fewer problems than those in parallel studies of cardiovascular diseases.

The specificity of the ethnic-group findings renders difficult an interpretation based solely on diagnostic factors. The comment from the 1921 report cited earlier might be elaborated to ask why death certification practices for ethnic groups should vary also by sites and in some instances discriminate by sex as well. A few hypotheses on the role of diagnostic factors for selected sites and ethnic groups might be entertained. The possible greater use of X rays among Jews (*50*) would favor the belief that as a group they received better medical care, but this leaves unexplained their pattern of excesses and deficits in risk by site. The mores of certain foreign-born groups might encourage less frequent examination of the reproductive organs and lead to underdiagnosis of these sites. Even if these conjectures had some substance, the basic question would remain one of arithmetic. Is it plausible that the substantial deficit in breast cancer among Italian migrants, for example, could be completely accounted for in this manner? Since death certificates offer one of the few feasible means for gathering the very large number of observations required in ethnic-group studies, queries to physicians signing death certificates should be encouraged, for the collection of more information on diagnostic methods and distinctions in histologic type. Utilization of cancer register data in states with large foreign-born populations could also place closer limits on the degree to which diagnostic artifacts might have influenced the ethnic-group results.

Systematic review of the ethnic-group results in the context of the total epidemiologic evidence for individual sites would be outside the scope of this paper. Some features of interest will be noted, but the comments are episodic rather than exhaustive. They are intended to outline some points of consistency between these results and other population characteristics for the sites in question and also to indicate how the ethnic-group data may contribute to clarification of possible anomalies in the American cancer incidence and mortality experience. It should be stressed that not all the ethnic-group findings can be reconciled with existing knowledge. Other applications will undoubtedly suggest themselves to the reader.

The range among migrant groups in mortality risks for all sites, up to 30 to 40 percent for males, warrants attention in view of the suggestion advanced by Goodhart (*51*) and others that the over-all cancer risk is dependent on host factors, with specific exposures to carcinogens merely determining the relative frequency of affected sites. In support of this thesis, Goodhart described the more uniform geographic distribution of cancer mortality for all sites than for specific sites which was observed in England and Wales. He made clear that he had more in mind than the well-known mathematical property of a statistical average or total being more stable than any of its component parts. In commenting on a somewhat related theory developed by Peller (*52*) on antagonistic action of different forms of cancer, Dorn and Cutler (*53*) demonstrated

that a compensatory balancing of the risks by site did not occur in regional comparisons of the 10-city incidence data; for example, the higher skin-cancer rates in the South were not offset by a decrease of cancer of other organs or tissues. Furthermore, such compensatory balancing has not been evident in unpublished U.S. regional tables of age-adjusted cancer mortality (*54*). The data for migrants follow the regional distributions in failure to support theories of compensatory effects, unless the proponents choose to regard 30 to 40 percent differences as trivial. To accommodate the findings on migrants within the framework of a host-factor determinant theory would require that the genetic effects differ among ethnic groups. Evidence for the latter is lacking, though in principle this postulate might be tested by the observation of whether the rates for all sites remain unchanged among each of the foreign-born groups and continue to differ among themselves and their native-born issue.

Buccal cavity.—The high SMR for buccal cavity among migrants from Ireland and the low rate among those born in USSR may not be inconsistent with the association between cancer of this site and excess consumption of alcohol, as evidenced by the higher risks for persons engaged in the alcoholic beverage trade (*55*). Impressions of an undue concentration of the Irish in this trade and of their above-average consumption of alcohol are not easy to document and such conditions may no longer exist. The most convincing evidence on the use of alcohol comes from mortality and mental hospital sources. Davis (*6*) was impressed with the extremely high mortality from alcoholism among the Irish in Boston as of 1900. Malzberg (*56*, *57*), in studying first admissions for alcoholic psychoses to New York State mental hospitals, 1929–31, noted that the Irish of both sexes had far higher admission rates than any other foreign-born group. Haggard and Jellinek (*58*) in a later publication, expanding on these same New York State data, showed the very low admission rates for Jews and summarized the literature on the rarity of alcoholic mental disorders and other alcoholic diseases among Jews.

The flaw in this line of speculation, which remains to be resolved, is that the buccal-cavity and pharynx rates have also been high in Ireland (*24*), where the rate of alcohol consumption has been lower than in most other European countries or in the United States (*59*). Increased consumption among Irish migrants may have been due to an improved standard of living and the ability to purchase liquor.

Esophagus.—The occupational mortality data, reinforced by retrospective studies of diagnosed cases and controls (*60*), have also suggested that alcohol has a role in cancer of the esophagus so that the comments on cancer of the buccal cavity among Irish migrants would apply to this site as well. In fact, one reason for alluding to a possible connection between the observations on this ethnic group and those on alcohol consumption is that the site distribution of excess risks among the Irish (buccal cavity, esophagus, intestines and rectum, but not stomach to the

the same degree) precisely parallels that implicated in studies of persons in the alcoholic beverage trades (*55*).

The presence of the foreign-born and their immediate descendants in large cities could account for part, but not all, of the high urban-rural ratios of mortality from cancer of the esophagus for both sexes in the United States, which are greater than that experienced in Denmark (*61*), England (*62*), and Finland (*32*); only Norway has reported urban-rural ratios for this site comparable to those for the United States (*48*). The foreign-born may have contributed in a similar manner to the American socioeconomic class gradient for this disease (*53*). The American gradients for males appear more pronounced than those reported in England (*55*) and Denmark (*63*). Unpublished 10-city data for males which suggest a somewhat steeper gradient in the four northern cities, where the foreign-born population is concentrated, than in the southern and western cities lend support to this conjecture. The data on foreign-born would not be helpful in accounting for the less marked urban-rural and social class gradients among American females. The same type of sex differences in these relationships appears in the data from the other countries. Why migrant males, but not females, should almost uniformly have experienced higher risks than those remaining in the home country remains a question worthy of further study.

Since an association of cancer of the esophagus with Plummer-Vinson disease in Swedish women has suggested a connection with nutrition, one may go further to question whether the ethnic-group variation for this disease reflects dietary and nutritional effects arising from food customs prevailing in the countries of origin and continued in the United States by the importation of these customs.

Stomach.—Unlike esophagus, there was no indication that the excess stomach-cancer risks among the foreign-born enhanced the strong inverse relationship with social class for this site. The relationship was just as marked in the southern and western cities covered in the 10-city surveys as in the northern cities. This is not surprising, since the social class gradient has figured prominently in reports from England (*55*) and Denmark (*63*) where there has been no influx of migrants.

The regional variation within the United States in the urban-rural ratios for the 1950 death rates for cancer of the stomach has been described (4 geographic divisions with ratios greater than unity and 4 others with ratios less than unity) (*19*). At the time, the lack of an orderly pattern weighed against the presumption of an association with urban or rural environmental factors. It is now clear that regional variation in urban-rural ratios can be resolved by controlling for nativity and that when this is done a consistent picture of trivial urban-rural differences in mortality from cancer of the stomach will emerge.

Regional data for the stomach, intestines, and rectum revealed differences between northern and southern States of about the same order for each site (*19*). Since the stomach does not resemble the intestines and

rectum in other characteristics including social class variation, such a finding might not have been anticipated from *a priori* considerations. Control of country of birth alters the picture profoundly. The North-South differences for stomach cancer were greatly diminished when comparisons were restricted to native whites and remained essentially unaltered for intestines and rectum, so that the revised regional pattern dovetails more closely with the other epidemiologic characteristics of these diseases.

Age-adjusted death rates per 100,000 population, 1949–51:

	All whites			Native whites		
	Stomach	Intestines	Rectum	Stomach	Intestines	Rectum
Males						
9 Northern States	22.7	17.5	10.4	18.1	16.6	9.2
13 Southern States	15.8	10.0	4.2	15.2	9.7	3.9
Ratio: S/N	.70	.57	.40	.84	.58	.42
Females						
9 Northern States	12.4	18.7	6.9	9.7	18.8	6.7
13 Southern States	9.1	12.5	3.7	8.9	12.3	3.6
Ratio: S/N	.73	.67	.54	.92	.65	.54

The nativity data may also provide a partial explanation for the extremely rapid rate of decline in stomach-cancer mortality in the United States (*19*, *64*, *65*). In the population of the United States, the proportion of foreign-born over age 40 (the ages at which stomach cancer becomes a frequent cause of death) has diminished over the years so that the contribution of these high-risk groups to the over-all national rate has become progressively less important. This does not imply that a drop has not occurred among the U.S. native whites, but suggests that the rate of decline for the latter may conform more closely to recent trends in several other countries.

The ranking by ethnic groups displayed many points of consistency with the international comparisons of stomach-cancer mortality and would support the inferences from the latter sources of greater risks in countries in the more northerly latitudes. While the foreign-born were not distributed in the exact order expected from location of home country, migrants from the southernmost country, Italy, did exhibit the lowest risk.

Studies in the United States on familial aggregation of stomach cancer have led to divergent estimates of the excess risks among relatives of diagnosed persons. Graham and Lilienfeld recently reviewed the literature (*66*) and noted that the estimates ranged from a 70 percent excess reported by Woolf from Utah (*67*) to a fourfold one noted by Macklin in Ohio (*68*). In a more recent article which used age-specific proportionate death rates for the general population to compute an "expected" number of deaths, Macklin (*69*) reported on another Ohio series which indicated an 80 percent excess among relatives. Since the proportion of foreign-born is negligible in Utah and substantial in Ohio, and in Ohio the representation of foreign-born among recently diagnosed stomach-cancer patients may have been diminishing, it might be worth inquiring

into the proportion of index cases, either foreign-born or those with foreign-born relatives, who were included in the Macklin material, particularly the earlier series. Her computations of "expected" deaths were based on the mortality of all Ohio whites, which would reflect preponderantly native-white experience. The procedure could have seriously underestimated "expected" deaths with a resultant overestimate of a familial excess, if substantial numbers of foreign-born had been included in the index case series. Control for ethnic group might do much to reconcile divergent findings on familial aggregation. Certainly the ethnic group differences lend cogency to Graham and Lilienfeld's remarks that, "A wide variety of socioeconomic, ethnic, racial or other cultural differences between subpopulations in the communities examined and the general population of the state could obscure any differences that distinguish the cancer from the non-cancer groups. On the other hand, any difference observed in such instances could be a product of difference between the study group and the state population rather than between a gastric cancer and non-gastric cancer population."

More generally, among migrant populations and for sites in which there is reason to suspect ethnic group differences in risk, control for nativity will need to be introduced into studies on factors other than familial aggregation, including such topics as geographic and social class variation. In retrospective investigations of diagnosed patients and controls, this would call for adding country of birth to items to be matched or otherwise accounted for in an analysis.

These comments may also be directed to the obvious hazards involved in applying the experience of one country as a standard for a group in another country. While it turned out that Mustacchi *et al.* (*70*), in using the U.S. 10-city breast-cancer incidence to determine an expected number of second breast cancers among female breast-cancer patients treated at an Italian clinic, erred on the conservative side in minimizing the difference between the numbers observed and expected, yet the reverse would often be true and such practices could not be generally recommended.

Intestines.—Apart from the higher rates for Irish males already mentioned, the more stable ethnic-group results for intestines and rectum would be in accord with other contrasts in behavior of epidemiological characteristics for the lower and upper digestive tract. The resemblance in the distributions for intestines and rectum is not surprising in view of the rather artificial anatomical division of these two sites.

Lung and bronchus.—The lung-cancer data on migrants from England agree in essence with the reports from New Zealand (*10*) and South Africa (*71*). The American material for several migrant groups from countries experiencing higher and lower lung-cancer death rates indicates a displacement in rates in either direction from those descriptive of the country of origin to a position intermediate between the home and host countries. The findings support inferences on the role of environmental exposures early in life as an important determinant of subsequent mortality

from lung cancer. While no specific information on the foreign-born as to age of entry or duration of residence in this country was available for tabulations corresponding to those of Eastcott which demonstrated larger differences for persons migrating after age 30, the great majority of U.S. migrants (other than persons born in Canada and Mexico) arrived in this country at least 30 years prior to death, before restrictions on immigration went into force.

Unpublished data (*72*), while not definitive, indicate minor differences between the native- and foreign-born with respect to current and lifetime smoking histories. Control of these factors would be unlikely to dispose of the more general nativity differentials in lung-cancer risk or of such extreme ethnic-group contrasts as those for Polish and Italian males, and in this respect would parallel the unsuccessful attempts to account for urban-rural differences in lung-cancer risk in this fashion (*73*). Even if such refinements as a count of the total amount of cigarettes consumed could be introduced on the presumption that migrants continued the frugal European practice of leaving shorter butts (*74–77*) [with consequent exposure to greater concentrations of tars (*77*) and benzo[*a*]pyrene (*78*)], the prospects of accounting for ethnic-group differences in terms of smoking group risks alone must still be regarded as remote.

In a general sense the ethnic-group data might be regarded as consistent with the universally observed urban-rural differences in that both suggest still unidentified environmental factors other than cigarette smoking. There is no need to appeal to observations on the foreign-born to account for the higher urban lung-cancer rates in the United States. The urban-rural differentials in England (*62*) and Finland (*32*) are of the same order of magnitude and differences in this respect are even more marked in Denmark (*61*) and Norway (*48*). At most, the excess risks among the foreign-born might exaggerate slightly the urban-rural differences observed here.

On the other hand the foreign-born experience may have accentuated the American socioeconomic class gradient for lung cancer. The class gradient was a prominent feature in the 10-city survey (*53*). The social class gradient for lung cancer was not pronounced in the English mortality data for 1949–53 (*55*) and had not been a feature of the data for 1921–23 and 1930–32 (*79*). The material from the Danish cancer register (*63*) also indicated a smaller social class gradient. The effect of the foreign-born on lung cancer figures is suggested in the 10-city data by the high ratios for the lowest income class in the northern cities where the foreign-born population is concentrated; the corresponding ratios in southern and western cities deviate less markedly from those for higher income classes.

While effects presumably associated with environmental factors other than smoking history are important and of interest in elucidating additional knowledge on lung-cancer epidemiology, it seems desirable to stress that the ethnic-group differences alone could not account for the smoking class differentials. Their magnitudes, on the order of twofold differences, are entirely too small to account for 10- to 60-fold differences in the latter

connection, as is true for many other factors known to be positively associated to a lesser degree with lung cancer (*80*). The gradients in risks by smoking class have been observed to prevail in both urban and rural populations (*81*), and one may confidently expect that examination of data for native-whites and foreign-whites would reveal a similar situation.

Breast.—The higher breast-cancer risks among women from Great Britain and other areas of northwestern Europe fitted into the same pattern delineated by the universal findings of an excess mortality among single and infertile married women (*48, 53, 82–85*). In the following table the foreign-born migrants are ranked according to the percentage of women over 35 years who were either single or had not borne a child as of the time of the 1940 population census, the latest available data on this subject (*86*).

TABLE 8.—Marital and child-bearing status of white women 35 years and over by country of birth, United States, 1940.

Country of birth	Percentage		
	Total single and "infertile"	Single	Ever-married with no live-born children ("*infertile*")
Ireland	32.6	16.9	15.7
Canada	30.2	10.5	19.7
England and Wales	25.6	8.4	17.2
Germany	24.0	7.9	16.1
Scandinavia	22.7	7.1	15.6
Mexico	13.7	5.8	7.9
Austria	11.7	2.9	8.8
Russia	10.9	3.3	7.6
Czechoslovakia	10.4	2.6	7.8
Poland	8.9	2.9	6.0
Italy	8.2	1.8	6.4

A dichotomy existed with respect to the data in table 8 between women born in Canada, England, Ireland, Germany, Scandinavia, and the United States on one hand and those from central and southern Europe on the other, which corresponded rather closely to the ethnic group distribution of the breast-cancer SMR's. The spread among ethnic groups was greater, however, than could be predicted from the marital status data alone; the several sources agree that the age-adjusted rates for married women run about 70 percent of those for single women, and since fertile married women predominate in all groups, the variation attributable to such factors could scarcely amount to more than 5 to 10 percent.

The contrast between the age-specific breast-cancer mortality for native- and foreign-born women with the cross-over in rates around age 50 as depicted in text-figure 3 may cast new light on the forces responsible for the shifts in risk at the time of menopause. Clemmesen (*87, 88*) in examining the Danish register data discovered that the incidence dropped between ages 45 to 49 and 50 to 54, with the rates resuming

their upward trend at older ages. Similar findings were later reported from England, Norway, and the United States (*48*, *53*, *89*). However, Dorn and Cutler commented on their inability to confirm completely Clemmesen's observation from the available American sources, some of which clearly showed the drop at menopause (Connecticut, Up-State New York, selected urban areas, 1937–39) while in others it was barely discernible (Iowa, selected urban areas, 1947–48). While the interpretation of this configuration of incidence rates as reflecting changes in endocrine function, presumably hormonal in origin, has gained wide acceptance, some investigators have expressed reservations. MacMahon (*90*) has questioned it on the grounds that "it seems unlikely that a menopausal change would be responsible for so temporary a reduction in susceptibility." He advanced another hypothesis—that the break in orderly age-progression of rates in data for a cross-section of the population at a given time represented in reality an increased susceptibility to breast cancer among younger cohorts of women arising from changing exposure to factor(s) related to reproductive history and breast-cancer incidence. In a subsequent paper MacMahon ruled out changes in fertility *per se* as a possible cohort factor and favored instead altered breast-finding practices as a working hypothesis for investigation (*91*). The difficulty is that a cohort interpretation of this nature would imply a rising trend in breast-cancer risk, and there has been little or no indication of this. The stability of the American breast-cancer mortality rates during the past half century has been the impressive feature and the breast is far less remarkable in displaying signs of cohort displacement of rates than such sites as the lung and stomach.

On re-examination of the Dorn and Cutler breast-cancer summary in light of the contrasts in nativity, one can now see that the menopausal break in incidence rates appeared in the American sources reporting on populations with substantial foreign-born components and was virtually absent from the Iowa data, where the foreign-born contribution is negligible. Differences in this respect between native- and foreign-born might still be regarded as producing cohort effects, as MacMahon has postulated, since the proportion of foreign-born is diminishing among successively younger cohorts of women; but if limited primarily to the menopausal ages these differences would not have led to large sustained increases in breast-cancer risk over a period of years. Confidence in the reality of this menopausal effect is enhanced by the internal consistency between the mortality experience and the various American incidence data. If substantiated by further work, a new frame of reference would be provided and the question to be posed would read, "Why does the break at menopausal age appear more prominently in migrant groups and foreign populations than among U.S. native whites?" This calls for lines of inquiry departing from the prevailing interpretation based on the role of endogenous factors and, needless to say, no ready explanation is at hand for the question as modified.

At first glance, the breast-feeding hypothesis advanced by MacMahon

offers attractive possibilities, since it seems to have been more prevalent among the foreign-born and negative associations of breast cancer with duration of breast-feeding and the more frequent occurrence of non-nursing mothers in series of breast-cancer patients have been observed (*92–95*). However, one may ask whether the American results on the relationship between nursing history and breast cancer arise from an indirect association with nativity or vice versa. In this connection a recent study by MacMahon and Feinleib (*96*), in which diagnosed cases and controls were matched by nativity in addition to other variables, is of interest. Contrary to earlier findings, no appreciable difference between breast cancer and comparison patients in regard to nursing histories emerged. While the authors suggested that previously reported relationships might be referable to intermediate variables (age, parity, and socioeconomic characteristics), other characteristics closely allied to ethnic groups and which may discriminate between foreign- and native-born women should not be neglected.

The American socioeconomic class gradient for breast cancer is not impressive, even though Dorn and Cutler (*53*) indicated this form of cancer occurred with somewhat greater frequency in higher income groups. The 10-city pattern resembled that reported for Denmark (*63*) and did not approach the gradient in the English mortality data reported by the Registrar General (*55*). The gradient in the 10-city data might have been enhanced by nativity effects; the standardized incidence ratios that follow indicate it was more pronounced in northern cities. In the series for southern and western cities which reflect mainly the experience of native whites, there was little or no evidence of an association with socioeconomic class:

	High				Low
	(1)	(2)	(3)	(4)	(5)
4 Northern cities	112	101	98	96	94
6 Southern and western cities	95	105	93	104	105

Cervix.—It has been noted that the low cervical-cancer SMR for migrants from the USSR agreed with many observations on the infrequent occurrence of this disease among Jews; the reasons for this remain unestablished, though the presence of a protective effect conferred by circumcision of the spouse has been hypothesized (*97*). Not all studies of diagnosed cases of cancer of the cervix and matched controls have uncovered a negative association with this factor (*38, 98*), and the difficulties of securing accurate information on circumcision status in epidemiological studies have been emphasized (*99*). It has been observed in another connection, however, that a negative association with circumcision would not suffice to account for the regional variation of this disease within the United States, where higher rates are experienced in the South (*42*). The suggestively low SMR's for Irish and Italian migrant women appear to reinforce the regional data in casting doubt on the adequacy or completeness

of the theory on protective effects of circumcision, since circumcision has not been widely practiced in these groups.

The high cervical-cancer risk among Mexican migrants seems quite consistent with the excess risks in the lowest socioeconomic classes noted in this country and elsewhere (*53, 55, 63*). Steiner (*1*) has reviewed some observations from Mexico which tend to confirm impressions of a much greater risk among these women.

The SMR for Irish migrants is intriguing, even though it did not meet the conventional test for statistical significance, since it followed the classical pattern of a deficit in risk among women married at advanced ages and among infertile women (*53, 97, 100–102*). The high proportion of Irish-born women in the United States who remained single or did not have children has already been indicated in the discussion on breast. The New York State marriage statistics for the 1930 decade also showed Irish and Scandinavian women to have the highest median ages, at first marriage, of the foreign-born groups reported on (*7*). The relationship with age at marriage and fertility did not hold consistently throughout these data and obvious counter-examples are apparent. The low-risk Italian and Russian groups had almost the lowest percentages of single and infertile women, and the Italians in particular married rather young.

Earlier attempts to account for regional variation in terms of factors apparently associated with cervical cancer such as marital status, age-at-marriage, socioeconomic status, prevalence of syphilis, etc., were not successful (*42*). In view of this, the inability to reconcile the North-South differences in cervical-cancer mortality by restriction of the comparison to native whites is worth noting; the excess in the southern States was just as large among native whites.

Age-adjusted death rates per 100,000 females, 1949–51:

	All whites		Native whites	
	Cervix	Other and unspecified uterus	Cervix	Other and unspecified uterus
9 Northern States	9. 4	9. 4	9. 7	10. 2
13 Southern States	11. 3	9. 5	11. 5	9. 5
Ratio: S/N	1. 20	1. 01	1. 19	. 93

Control of nativity, however, might resolve some anomalies in cervical-cancer incidence in New York City as compared to data from other areas—the cervix rates at ages over 50 being much lower in New York City in the absence of large differences at younger ages or for uterine corpus at any age (*42*). The foreign-born are heavily represented in the older population of New York City, and women from Ireland, Italy, and USSR, all with low risks for this site, constitute three of the city's most important ethnic groups. The absence of a cervical-cancer differential at younger ages in the New York City white population intimates that the lower risks evidenced among certain foreign-born groups may not be transferable to their native-born descendants.

Since the inverse relationship in liability to breast and cervical cancer has been observed over a wide range of group contrasts, its absence from the data for Italian and possibly USSR migrants commands interest. Why these groups should depart from the conventional pattern remains obscure, and in studies of these two sites priority might well be assigned to this particular aspect of the ethnic-group data.

Corpus.—While deaths certified to "uterus" without further qualification undoubtedly include some attributable to cervix as well as those due to cancer of the uterine corpus, the SMR's for "other uterus" reflect in large measure the experience for corpus. The more compressed scale of ethnic-group variation for corpus in contrast to cervix would be in line with the general picture of greater variability for the latter site and would support the presumption of a greater role for endogenous factors in the development of corpus cancer.

Ovary.—Possibly the low SMR for Italian women could be singled out as diverging significantly from the total national experience for ovarian cancer. This would be in accord with the lack of evidence on an important role for specific environmental factors and the impression that the distribution of the disease is influenced by physiological and hormonal aspects of the female reproductive cycle. Too much should not be made of the higher risk for USSR females, though some connection might exist with the possibly greater exposure of Jewish women to diagnostic and therapeutic radiation procedures (*50*).

Prostate.—Even though the SMR's for four ethnic groups deviated significantly at the 1 percent level on the low side, the degree of variation for prostate was not overly impressive and was more reminiscent of that obtained for breast, ovary, intestines, and rectum than for esophagus, stomach, and lung. Evidence for the effect of environmental factors is lacking. No occupational exposures have been incriminated (*103*), and the relatively modest urban-rural and geographic variations in risk among whites (*1*, *20*, *25*) would not suggest the influence of exogenous agents. Also, carcinoma of the prostate develops late in life, which would imply under these circumstances extremely long induction periods. On the other hand, pronounced variations in risk exist among whites, Negroes, and orientals (*25*, *53*, *104*, *105*). This might lead to speculation as to whether the present ethnic-group differences reflect in a more dilute form effects of endogenous factors governing the racial distribution of prostatic cancer.

Bladder.—The results for bladder were most provocative. Among males this site displayed minimum ethnic-group variability. Of the known environmental carcinogens, exposure to the aromatic amines (*82*, *106*) would be the one most relevant in this context. There is no reason to believe that the groups varied importantly in this regard, so that the lack of variability among males might have been anticipated on *a priori* grounds. The situation among females was in direct contrast, with evidence of substantially lower rates among several groups of foreign-born women. No other etiologies have yet been proposed which could

account for this female distribution of SMR's, and there is left a strong intimation that gaps in knowledge about other possible etiologies for this site remain to be filled.

Leukemia.—The above-average mortality from leukemia among USSR migrants was in accord with other American studies dealing with the frequency of this disease among Jews. Ionizing radiation is the one firmly established leukemic agent (*106–111*), and it might be said that a greater exposure of Jews to diagnostic and therapeutic radiation would be in the right direction to be compatible with the ethnic-group observations. With this exception, and possibly that of Mexicans, whose risks resemble those of American Indians (*37*), the range of ethnic-group variation was small. The data do not suggest the operation of other environmental factors which discriminate among ethnic groups.

Lymphomas.—There is enough uncertainty about the epidemiologic characteristics of the lymphomas to discourage conjectures about the compatibility of ethnic-group results with other data. Since excess risks for USSR migrants and Jews persisted within all the major subtypes of leukemia, it is not surprising to observe that the same phenomenon for USSR migrants carried over to the several lymphoma categories as well.

From the site-by-site review, one tentative generalization emerges. The ethnic-group variation appears most marked in those sites for which there is some reason to believe that the pattern of risk could be associated with personal habits and customs, such as dietary practices, which migrants could bring with them from their native countries. The sites, for which work to date would favor implication of either endogenous factors or factors in the physical environment not readily transplantable with the migrants, display less variability.

One weakness in the present results arises from the restriction to contrasts of the foreign-born with native-born of any parentage. This has foreclosed for the time being review of ethnic-group gradients in risk from foreign-born to native-born of foreign parentage to native-born of foreign parentage to native-born of native parentage. Such information, while not subject to an unequivocal interpretation, would still be helpful in assessment of the situation for specific sites and in decisions reached as to whether the next round of studies should concentrate on endogenous or exogenous factors.

It seems undebatable that ethnic-group differences, when combined with other information, have a role to play in the selection and formulation of hypotheses for testing. They open new vistas for American investigators and may place them in an advantageous position to plan and conduct further probing studies. However, ethnic-group variation is

not an unmixed blessing, since it represents a potential complicating factor in American studies of geographic variation and familial aggregation.

Further investigation in this area is warranted and several lines of inquiries could be initiated to exploit and to enlarge on them. These include:

(*a*) A review of cancer deaths registered during years close to the 1930 and 1940 population censuses in the Northeastern and North Central States with the highest concentrations of foreign-born population, to determine mortality by ethnic group and degree of nativity for specific sites. This would permit evaluation of time trends in ethnic-group risks.

(*b*) To remedy deficiencies arising from the deletion of the parentage item from the last revision of the standard death certificate, a program should be initiated to determine parentage of native-born persons dying from cancer, by query of relatives.

(*c*) Such a query program might be expanded to collect for some sites information on the decedents' age of entry into the United States. This would permit examination of differentials in risk among persons migrating to the United States early or late in life in a manner similar to that described by Eastcott in New Zealand (*10*). It might be possible to utilize the records of the Immigration and Naturalization Service in this connection.

(*d*) The general population mortality data could be supplemented by long-term follow-up of cohorts of insured persons. One such group of males, World War I veterans holding Government life insurance policies, are currently under observation (*112*), and the foreign-born among them must have arrived in the United States at an early age. Cohort studies might make feasible the collection of information on other characteristics thought to be pertinent, but which are not covered by the conventional sources of mortality data.

(*e*) Collaborative studies with investigators in countries of origin, employing retrospective and/or prospective techniques, might be attempted to trace and identify high- and low-risk groups for specific sites within the parent populations and to determine whether and how the effects have carried over to their emigrants. Such cross-checks would strengthen inferences on the causal nature of the observational associations over those possible from a single-study setting. The steps outlined to develop further sources of ethnic-group information need not delay the pursuit of collaborative studies. The data at hand on ethnic-group effects for such sites as esophagus and stomach now warrant a program of retrospective and forward studies to identify the factors contributing to international and ethnic-group variation.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
	Foreign-born whites, total											
All sites (140–205)												
Observed	20201	8229	6256	1743	3154	819	14262	6322	3999	1193	2278	470
SMR	124	114	139	129	120	146	106	103	104	119	111	110
Buccal cavity and pharynx (140–148)												
Observed	694	282	243	49	95	25	121	53	38	7	16	7
SMR	105	89	145	89	96	105	90	85	110	65	81	106
Digestive system (150–159)												
Observed	9958	4000	3181	846	1556	375	6505	2893	1894	589	915	214
SMR	145	132	163	140	147	173	128	125	128	139	126	141
Esophagus (150)												
Observed	834	337	297	55	124	21	179	89	41	16	29	4
SMR	214	171	261	217	284	206	174	170	153	258	206	114
Stomach (151)												
Observed	3750	1382	1221	384	624	139	2012	840	630	198	281	63
SMR	179	163	202	185	173	182	190	188	206	194	171	169
Intestines (152–153)												
Observed	2074	884	626	172	316	76	1883	870	519	168	255	71
SMR	115	105	120	111	131	160	98	92	97	110	103	141
Rectum (154)												
Observed	1331	602	419	84	174	52	713	374	169	39	111	20
SMR	128	113	158	115	118	274	104	109	92	86	115	138
Pancreas (157)												
Observed	998	423	276	80	172	47	660	297	188	60	93	22
SMR	120	124	120	113	110	154	129	137	129	144	103	135
Respiratory system (160–165)												
Observed	3577	1580	1095	233	506	163	644	280	172	59	113	20
SMR	131	111	171	167	118	185	135	138	125	159	141	108
Lung and bronchus (162–163)												
Observed	3115	1380	949	205	437	144	573	248	154	51	101	19
SMR	133	113	174	176	116	192	139	140	129	162	145	120

Breast (170)												
Observed	43	24	9	2	7	1	2249	1033	580	178	393	65
SMR	118	129	107	61	152	67	83	77	80	103	99	102
Female genital organs (171–176)												
Observed							2317	1000	650	151	431	85
SMR							88	84	83	89	106	92
Cervix (171)												
Observed							727	305	212	40	147	23
SMR							90	88	89	89	106	63
Uterus, other and unspecified (172–174)												
Observed							751	299	228	55	134	35
SMR							78	71	75	79	105	101
Ovary (175)												
Observed							733	349	175	50	135	24
SMR							94	93	84	107	107	127
Male genital organs (177–179)												
Observed	1839	614	566	244	335	80						
SMR	88	78	90	103	93	95						
Prostate (177)												
Observed	1767	589	545	234	324	75						
SMR	89	80	91	101	95	93						
Bladder (181)												
Observed	812	352	214	68	140	38	229	97	60	18	42	12
SMR	99	89	96	127	108	182	67	61	62	75	84	126
Leukemia (204)												
Observed	621	263	177	70	87	24	398	176	116	33	61	12
SMR	107	119	103	133	77	111	112	112	122	109	98	103
Lymphomas (200–203, 205)												
Observed	682	303	183	63	103	30	431	207	110	30	75	9
SMR	116	121	114	147	92	149	123	137	110	115	125	81
All other sites												
Observed	1975	811	588	168	325	83	1368	583	379	128	232	46
SMR	104	103	109	102	102	98	97	100	93	115	97	77

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
	England and Wales											
All sites (140–205)												
Observed	1114	418	275	68	262	91	945	353	246	49	257	40
SMR	118	114	115	129	110	185	107	97	113	116	120	84
Buccal cavity and pharynx (140–148)												
Observed	45	21	8	2	12	2	5	3	2	—	—	—
SMR	118	130	91	91	135	95	57	81	105	—	—	—
Digestive system (150–159)												
Observed	545	209	132	37	120	47	403	146	112	26	105	14
SMR	136	135	126	156	123	244	117	101	131	146	131	80
Esophagus (150)												
Observed	32	16	5	1	7	3	15	2	5	1	7	—
SMR	150	165	85	111	179	333	221	61	333	500	467	—
Stomach (151)												
Observed	190	66	45	14	47	18	125	43	38	11	28	5
SMR	151	150	138	171	139	261	169	149	209	256	153	116
Intestines (152–153)												
Observed	122	45	38	10	23	6	133	58	27	10	33	5
SMR	117	102	136	167	103	146	103	99	87	156	122	88
Rectum (154)												
Observed	75	39	14	4	11	7	41	18	7	1	13	2
SMR	125	140	101	143	81	41	90	85	66	53	126	125
Pancreas (157)												
Observed	81	35	15	5	17	9	34	11	12	1	10	—
SMR	168	208	124	185	121	346	96	81	141	56	101	—

Respiratory system (160–165)												
Observed	184	76	45	11	38	14	48	21	13	—	13	1
SMR	128	117	143	224	107	200	149	171	167	—	155	48
Lung and bronchus (162–163)												
Observed	160	63	41	11	33	12	41	17	12	—	11	1
SMR	131	115	155	268	106	203	150	160	182	—	153	59
Breast (170)												
Observed	1	—	1	—	—	—	178	75	37	9	48	9
SMR	59	—	250	—	—	—	105	99	93	123	119	130
Female genital organs (171–176)												
Observed							169	66	38	5	48	12
SMR							102	100	89	71	119	122
Cervix (171)												
Observed							58	22	12	2	18	4
SMR							115	119	93	111	135	103
Uterus, other and unspecified (172–174)												
Observed							57	26	11	2	14	4
SMR							93	106	65	71	107	105
Ovary (175)												
Observed							42	14	11	1	13	3
SMR							90	70	99	59	106	150
Male genital organs (177–179)												
Observed	132	47	29	8	35	13						
SMR	96	100	80	82	96	159						
Prostate (177)												
Observed	128	46	29	8	34	11						
SMR	97	103	83	84	97	141						
Bladder (181)												
Observed	52	18	17	3	12	2	19	7	4	1	6	1
SMR	105	85	142	136	98	105	79	67	70	100	105	83
Leukemia (204)												
Observed	27	11	5	3	7	1	19	3	5	1	10	—
SMR	81	101	56	158	71	56	81	32	93	83	154	—
Lymphomas (200–203, 205)												
Observed	27	4	8	—	12	3	26	7	9	1	9	—
SMR	85	34	100	—	133	188	119	88	164	91	148	—
All other sites												
Observed	101	32	30	4	26	9	78	25	26	6	18	3
SMR	94	84	106	65	94	123	83	74	111	125	71	45

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
Ireland												
All sites (140–205)												
Observed	928	562	165	54	115	32	926	623	149	39	94	21
SMR	134	127	137	180	133	235	110	110	106	134	114	120
Buccal cavity and pharynx (140–148)												
Observed	64	44	13	1	4	2	15	12	1	—	2	—
SMR	224	227	295	83	129	400	181	203	83	—	286	—
Digestive system (150–159)												
Observed	473	291	88	25	55	14	455	306	74	21	44	10
SMR	162	156	168	187	155	259	137	136	132	168	142	152
Esophagus (150)												
Observed	53	32	10	3	7	1	15	11	2	1	1	—
SMR	315	274	357	500	467	500	205	208	182	500	200	—
Stomach (151)												
Observed	131	76	28	8	16	3	140	96	20	5	17	2
SMR	149	144	170	170	130	158	204	215	168	167	236	118
Intestines (152–153)												
Observed	128	82	25	7	11	3	145	93	27	9	12	4
SMR	161	156	177	200	134	250	113	102	132	205	114	190
Rectum (154)												
Observed	82	53	12	4	10	3	59	42	8	2	6	1
SMR	174	160	171	235	204	600	129	127	118	154	150	167
Pancreas (157)												
Observed	50	30	9	1	7	3	48	30	11	2	4	1
SMR	148	147	145	63	140	500	150	143	200	182	105	143

Respiratory system (160–165)												
Observed	112	66	19	5	21	1	36	17	10	2	5	2
SMR	98	82	121	179	159	53	122	89	200	182	152	250
Lung and bronchus (162–163)												
Observed	79	46	13	5	14	1	33	16	10	1	4	2
SMR	81	67	98	208	122	63	131	97	233	111	138	400
Breast (170)												
Observed	1	—	1	—	—	—	162	104	27	7	21	3
SMR	71	—	500	—	—	—	97	87	105	143	139	120
Female genital organs (171–176)												
Observed							130	95	16	4	9	6
SMR							84	91	59	83	59	176
Cervix (171)												
Observed							33	27	3	—	2	1
SMR							73	92	37	—	39	77
Uterus, other and unspecified (172–174)												
Observed							48	33	6	1	4	4
SMR							83	86	55	53	78	308
Ovary (175)												
Observed							39	27	6	2	3	1
SMR							86	85	87	154	65	167
Male genital organs (177–179)												
Observed	117	64	20	11	17	5						
SMR	125	118	108	200	132	208						
Prostate (177)												
Observed	113	64	17	11	16	5						
SMR	127	125	97	200	128	227						
Bladder (181)												
Observed	30	16	3	1	6	4	15	9	4	—	2	—
SMR	81	64	49	91	136	800	65	57	108	—	87	—
Leukemia (204)												
Observed	24	15	3	4	2	—	18	13	2	1	2	—
SMR	106	114	68	364	57	—	85	92	61	125	80	—
Lymphomas (200–203, 205)												
Observed	22	12	6	2	1	1	18	14	2	1	1	—
SMR	94	82	154	200	31	200	93	110	61	143	43	—
All other sites												
Observed	85	55	12	5	9	4	77	53	13	3	8	—
SMR	111	117	85	139	90	200	94	102	87	94	83	—

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
Norway												
All sites (140–205)												
Observed	515	85	134	163	122	11	392	71	107	103	104	7
SMR	114	111	128	114	99	172	111	108	116	101	119	149
Buccal cavity and pharynx (140–148)												
Observed	24	8	4	6	6	—	2	—	1	—	—	1
SMR	134	229	105	103	133	—	57	—	125	—	—	—
Digestive system (150–159)												
Observed	249	34	65	78	67	5	182	32	54	49	43	4
SMR	127	107	141	121	133	200	128	132	140	111	131	222
Esophagus (150)												
Observed	22	4	5	8	4	1	4	2	1	—	1	—
SMR	239	200	200	296	200	—	167	400	143	—	200	—
Stomach (151)												
Observed	121	15	34	35	35	2	75	10	29	20	13	3
SMR	189	172	231	157	202	222	236	213	341	187	173	750
Intestines (152–153)												
Observed	38	1	11	15	10	1	38	6	9	13	9	1
SMR	76	11	89	90	87	200	74	61	64	82	82	167
Rectum (154)												
Observed	29	6	8	7	7	1	15	2	3	2	8	—
SMR	109	107	133	90	100	500	86	56	64	43	190	—
Pancreas (157)												
Observed	24	5	2	10	7	—	21	6	5	6	4	—
SMR	100	139	38	135	96	—	144	273	135	136	98	—

Respiratory system (160–165)												
Observed	44	13	12	15	2	2	21	5	5	5	6	—
SMR	71	84	97	107	10	222	162	227	152	128	176	—
Lung and bronchus (162–163)												
Observed	37	10	10	13	2	2	19	4	5	5	5	—
SMR	70	75	96	112	12	250	168	211	172	156	161	—
Breast (170)												
Observed	—	—	—	—	—	—	63	14	17	19	13	—
SMR	—	—	—	—	—	—	97	96	105	112	80	—
Female genital organs (171–176)												
Observed							63	14	12	11	26	—
SMR							99	108	72	66	159	—
Cervix (171)												
Observed							18	7	—	4	7	—
SMR							98	179	—	95	132	—
Uterus, other and unspecified (172–174)												
Observed							21	1	6	2	12	—
SMR							86	22	83	29	222	—
Ovary (175)												
Observed							21	4	6	4	7	—
SMR							118	98	146	93	137	—
Male genital organs (177–179)												
Observed	81	11	23	26	20	1						
SMR	116	139	133	99	113	111						
Prostate (177)												
Observed	81	11	23	26	20	1						
SMR	120	151	138	102	116	111						
Bladder (181)												
Observed	22	4	5	7	6	—	6	—	2	2	1	1
SMR	101	100	91	123	97	—	64	—	71	80	42	1000
Leukemia (204)												
Observed	17	—	8	4	5	—	10	2	3	3	2	—
SMR	102	—	222	74	98	—	109	125	143	100	80	—
Lymphomas (200–203, 205)												
Observed	16	1	5	7	2	1	11	1	3	3	4	—
SMR	105	36	161	163	42	500	124	59	136	115	167	—
All other sites												
Observed	62	14	12	20	14	2	34	3	10	11	9	1
SMR	118	165	101	117	98	222	89	48	102	98	88	143

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
	Sweden											
All sites (140–205)												
Observed	899	142	285	235	214	23	633	98	199	199	160	7
SMR	109	99	121	103	107	162	102	75	95	131	109	58
Buccal cavity and pharynx (140–148)												
Observed	19	3	7	7	2	—	11	1	6	3	—	1
SMR	59	48	80	76	27	—	164	71	316	176	—	500
Digestive system (150–159)												
Observed	458	78	144	111	113	12	307	44	99	103	59	2
SMR	129	129	138	109	136	218	114	83	113	156	103	43
Esophagus (150)												
Observed	29	6	12	5	5	1	10	2	1	5	2	—
SMR	167	158	203	119	152	500	200	167	63	500	182	—
Stomach (151)												
Observed	210	31	66	51	54	8	107	14	33	40	19	1
SMR	181	181	201	144	188	400	175	130	171	245	141	91
Intestines (152–153)												
Observed	96	17	28	27	22	2	74	6	26	25	16	1
SMR	105	99	100	102	116	154	76	28	82	105	84	67
Rectum (154)												
Observed	42	11	10	3	17	1	25	9	6	4	6	—
SMR	86	101	72	24	149	200	75	115	57	56	80	—
Pancreas (157)												
Observed	43	8	11	16	8	—	40	4	14	14	8	—
SMR	101	121	92	138	68	—	145	82	167	212	113	—

Respiratory system (160–165)												
Observed	92	12	28	24	24	4	29	3	9	9	8	—
SMR	85	47	95	109	82	211	121	67	120	164	136	—
Lung and bronchus (162–163)												
Observed	83	11	25	22	21	4	22	1	7	7	7	—
SMR	90	51	100	121	82	235	108	26	108	152	140	—
Breast (170)												
Observed	2	1	—	—	1	—	105	15	32	30	27	1
SMR	133	333	—	—	333	—	90	56	87	120	102	67
Female genital organs (171–176)												
Observed							99	14	24	28	33	—
SMR							87	60	63	116	125	—
Cervix (171)												
Observed							25	1	11	7	6	—
SMR							78	16	103	117	71	—
Uterus, other and unspecified (172–174)												
Observed							39	3	8	10	18	—
SMR							86	34	49	96	200	—
Ovary (175)												
Observed							31	10	4	11	6	—
SMR							100	143	43	172	77	—
Male genital organs (177–179)												
Observed	148	17	57	42	32	—						
SMR	112	93	151	100	101	—						
Prostate (177)												
Observed	145	16	56	41	32	—						
SMR	114	92	153	100	105	—						
Bladder (181)												
Observed	35	5	13	8	8	1	6	—	1	1	4	—
SMR	87	61	107	89	76	200	33	—	17	26	95	—
Leukemia (204)												
Observed	32	5	6	15	6	—	19	4	8	3	4	—
SMR	108	125	71	176	74	—	112	121	167	70	95	—
Lymphomas (200–203, 205)												
Observed	24	5	7	3	7	2	16	4	2	5	5	—
SMR	90	111	93	43	96	400	102	143	42	132	125	—
All other sites												
Observed	89	16	23	25	21	4	71	13	18	17	20	3
SMR	94	107	84	92	92	190	102	109	81	101	116	188

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
Germany												
All sites (140–205)												
Observed	2428	838	827	364	303	96	1983	726	710	259	231	57
SMR	122	113	126	125	127	140	106	100	108	111	118	89
Buccal cavity and pharynx (140–148)												
Observed	75	32	25	10	6	2	9	3	4	—	2	—
SMR	93	98	104	84	67	69	47	41	65	—	105	—
Digestive system (150–159)												
Observed	1265	416	444	194	161	50	951	331	363	134	95	28
SMR	148	134	153	148	164	185	125	115	132	131	128	112
Esophagus (150)												
Observed	90	37	31	13	8	1	25	8	10	4	3	—
SMR	198	189	199	250	205	83	163	119	196	267	214	—
Stomach (151)												
Observed	491	157	163	92	64	15	315	95	132	42	33	13
SMR	182	179	176	202	188	155	189	165	218	166	190	210
Intestines (152–153)												
Observed	260	78	102	32	34	14	291	103	112	41	27	8
SMR	114	88	131	94	151	230	102	89	113	111	108	96
Rectum (154)												
Observed	185	56	75	26	19	9	95	47	30	7	8	3
SMR	148	100	198	165	140	375	97	111	92	65	82	125
Pancreas (157)												
Observed	113	46	31	15	17	4	73	31	26	8	7	1
SMR	114	135	94	102	123	118	98	116	98	79	78	40

Respiratory system (160–165)												
Observed	313	134	98	27	39	15	82	29	22	15	15	1
SMR	113	101	130	103	113	165	121	116	94	174	195	36
Lung and bronchus (162–163)												
Observed	264	112	84	23	32	13	73	28	20	12	13	—
SMR	112	99	133	108	107	173	126	131	99	164	194	—
Breast (170)												
Observed	11	4	3	1	2	1	336	140	115	33	40	8
SMR	268	222	273	167	500	500	96	91	100	87	112	90
Female genital organs (171–176)												
Observed							298	118	96	37	36	11
SMR							88	89	81	101	101	89
Cervix (171)												
Observed							98	43	34	7	10	4
SMR							101	113	101	75	85	87
Uterus, other and unspecified (172–174)												
Observed							93	26	35	16	14	2
SMR							71	54	69	101	120	43
Ovary (175)												
Observed							91	45	18	12	12	4
SMR							98	110	62	125	112	167
Male genital organs (177–179)												
Observed	303	79	107	65	42	10						
SMR	97	85	95	117	112	82						
Prostate (177)												
Observed	286	73	102	62	40	9						
SMR	96	83	94	114	110	76						
Bladder (181)												
Observed	112	47	35	13	14	3	42	15	19	2	3	3
SMR	109	111	103	113	113	111	80	75	100	33	55	188
Leukemia (204)												
Observed	69	29	22	9	6	3	44	13	17	7	7	—
SMR	101	129	95	85	61	120	91	70	110	101	123	—
Lymphomas (200–203, 205)												
Observed	67	19	16	12	15	5	53	17	16	8	9	3
SMR	106	77	82	143	170	238	121	105	106	138	170	214
All other sites												
Observed	213	78	77	33	18	7	168	60	58	23	24	3
SMR	94	98	102	96	66	69	86	90	83	89	103	32

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
					Poland							
All sites (140–205)												
Observed	2129	894	1027	67	85	56	1258	632	515	43	44	24
SMR	145	118	176	153	153	194	105	103	106	134	109	102
Buccal cavity and pharynx (140–148)												
Observed	72	21	48	—	1	2	10	5	3	1	—	1
SMR	119	63	218	—	50	167	88	81	70	333	—	333
Digestive system (150–159)												
Observed	1031	426	512	30	40	23	572	279	241	20	17	15
SMR	166	135	202	154	180	207	132	125	136	159	125	190
Esophagus (150)												
Observed	131	38	89	2	1	1	12	5	6	1	—	—
SMR	336	180	574	250	100	167	138	102	194	500	—	—
Stomach (151)												
Observed	413	178	196	12	18	9	176	79	77	5	10	5
SMR	225	202	254	182	240	231	209	189	221	172	345	263
Intestines (152–153)												
Observed	173	76	75	10	8	4	142	79	56	2	2	3
SMR	104	87	112	204	163	167	85	87	86	43	43	115
Rectum (154)												
Observed	111	54	50	2	2	3	75	40	29	3	2	1
SMR	115	97	143	87	67	333	125	120	128	214	118	125
Pancreas (157)												
Observed	106	42	49	1	10	4	62	35	21	4	1	1
SMR	143	115	161	42	313	250	144	166	117	308	56	111

Respiratory system (160–165)												
Observed	492	216	223	13	23	17	60	32	23	3	2	—
SMR	183	137	247	245	232	340	148	164	131	273	133	—
Lung and bronchus (162–163)												
Observed	434	198	187	12	21	16	54	29	20	3	2	—
SMR	188	145	241	267	244	372	150	170	131	273	133	—
Breast (170)												
Observed	3	1	—	—	2	—	168	99	50	8	10	1
SMR	100	53	—	—	—	—	68	73	53	136	120	27
Female genital organs (171–176)												
Observed							212	90	107	5	7	3
SMR							86	73	102	85	80	56
Cervix (171)												
Observed							71	24	43	—	4	—
SMR							95	67	133	—	133	—
Uterus, other and unspecified (172–174)												
Observed							74	25	44	2	2	1
SMR							83	58	112	87	77	50
Ovary (175)												
Observed							63	38	19	3	1	2
SMR							85	97	66	167	36	167
Male genital organs (177–179)												
Observed	127	54	59	6	3	5						
SMR	76	72	80	88	45	135						
Prostate (177)												
Observed	125	50	59	6	3	7						
SMR	79	70	85	91	47	189						
Bladder (181)												
Observed	69	36	26	1	5	1	12	7	4	—	1	—
SMR	91	87	91	59	185	83	42	47	35	—	111	—
Leukemia (204)												
Observed	56	19	29	5	1	2	48	25	19	2	1	1
SMR	108	81	127	278	43	182	157	163	154	200	77	167
Lymphomas (200–203, 205)												
Observed	72	35	28	5	2	2	49	31	17	—	1	—
SMR	135	133	128	357	83	167	152	195	126	—	77	—
All other sites												
Observed	207	86	102	7	8	4	127	64	51	4	5	3
SMR	122	104	144	130	118	91	104	108	99	114	111	94

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
Czechoslovakia												
All sites (140–205)												
Observed	630	148	341	78	40	23	446	115	236	50	32	13
SMR	133	104	148	130	151	173	106	93	109	104	161	113
Buccal cavity and pharynx (140–148)												
Observed	26	7	15	1	1	2	3	2	1	—	—	—
SMR	138	113	172	42	100	400	71	154	53	—	—	—
Digestive system (150–159)												
Observed	347	73	192	46	24	12	226	61	114	32	13	6
SMR	171	122	190	171	220	235	142	133	140	156	183	136
Esophagus (150)												
Observed	37	7	23	2	4	1	6	2	3	1	—	—
SMR	322	179	390	182	1333	333	222	182	214	500	—	—
Stomach (151)												
Observed	139	28	76	21	10	4	86	26	41	10	8	1
SMR	222	168	245	226	270	222	264	302	250	204	500	91
Intestines (152–153)												
Observed	70	15	38	12	3	2	53	15	26	8	1	3
SMR	130	90	142	174	120	167	89	80	88	107	42	200
Rectum (154)												
Observed	44	14	22	3	3	2	26	8	12	4	1	1
SMR	150	133	162	94	188	400	127	119	118	182	100	250
Pancreas (157)												
Observed	21	4	13	2	1	1	22	6	10	3	2	1
SMR	87	58	109	65	63	143	138	136	123	143	222	200

Respiratory system (160–165)												
Observed	97	28	56	5	5	3	16	2	9	2	3	—
SMR	132	99	168	85	119	158	108	50	115	111	429	—
Lung and bronchus (162–163)												
Observed	84	25	46	5	5	3	14	1	8	2	3	—
SMR	134	103	163	100	135	188	109	29	116	133	429	—
Breast (170)												
Observed	1	—	—	1	—	—	63	21	34	3	3	2
SMR	111	—	—	500	—	—	77	78	83	37	77	125
Female genital organs (171–176)												
Observed							69	13	39	6	8	3
SMR							83	53	87	75	205	130
Cervix (171)												
Observed							25	6	12	2	4	1
SMR							100	85	88	95	308	125
Uterus, other and unspecified (172–174)												
Observed							27	5	14	3	3	2
SMR							86	57	81	94	250	222
Ovary (175)												
Observed							14	2	10	1	1	—
SMR							59	26	83	43	83	—
Male genital organs (177–179)												
Observed	50	15	24	8	—	3						
SMR	79	97	76	75	—	136						
Prostate (177)												
Observed	49	14	24	8	—	3						
SMR	80	97	79	76	—	136						
Bladder (181)												
Observed	21	3	9	3	4	2	2	1	—	—	—	1
SMR	90	38	79	125	308	400	20	32	—	—	—	500
Leukemia (204)												
Observed	24	3	14	5	1	1	9	1	6	1	1	—
SMR	144	71	159	227	91	250	84	32	113	71	167	—
Lymphomas (200–203, 205)												
Observed	18	7	7	3	1	—	12	4	7	1	—	—
SMR	111	146	86	158	100	—	108	125	121	83	—	—
All other sites												
Observed	46	11	24	6	4	1	46	10	26	5	4	1
SMR	84	72	87	85	129	50	104	83	114	94	167	63

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
					Austria							
All sites (140–205)												
Observed	938	474	283	68	90	23	659	421	126	39	53	20
SMR	119	102	152	165	120	113	104	111	81	129	103	138
Buccal cavity and pharynx (140–148)												
Observed	25	5	12	2	6	—	7	4	3	—	—	—
SMR	76	24	171	118	214	—	111	103	214	—	—	—
Digestive system (150–159)												
Observed	458	224	158	32	37	7	295	190	61	16	19	9
SMR	138	115	195	174	121	86	127	135	108	132	104	176
Esophagus (150)												
Observed	39	15	19	3	2	—	2	—	1	—	1	—
SMR	196	118	396	429	154	—	43	—	10	—	333	—
Stomach (151)												
Observed	166	68	59	20	15	4	85	52	20	7	4	2
SMR	168	125	237	317	147	143	185	195	179	241	100	167
Intestines (152–153)												
Observed	93	59	25	2	5	2	95	58	21	3	10	3
SMR	104	109	116	43	72	111	105	101	101	70	161	188
Rectum (154)												
Observed	65	34	24	1	5	1	33	24	6	2	1	—
SMR	123	99	218	43	119	143	102	115	83	143	42	—
Pancreas (157)												
Observed	49	26	13	5	5	—	39	25	10	2	—	2
SMR	124	118	135	227	111	—	170	185	179	167	—	500

Respiratory system (160–165)												
Observed	188	102	51	14	15	6	28	20	3	3	1	1
SMR	134	111	185	304	118	194	130	163	55	250	50	167
Lung and bronchus (162–163)												
Observed	168	91	47	12	13	5	27	19	3	3	1	1
SMR	140	115	199	308	116	185	141	174	61	300	59	167
Breast (170)												
Observed	2	2	—	—	—	—	100	63	18	6	10	3
SMR	125	182	—	—	—	—	77	76	60	109	100	136
Female genital organs (171–176)												
Observed							113	70	24	5	11	3
SMR							89	93	73	91	107	91
Cervix (171)												
Observed							30	14	11	—	4	1
SMR							78	65	107	—	111	83
Uterus, other and unspecified (172–174)												
Observed							29	20	5	2	1	1
SMR							63	75	40	91	30	83
Ovary (175)												
Observed							45	31	5	2	6	1
SMR							117	130	55	125	182	143
Male genital organs (177–179)												
Observed	69	29	19	6	12	3						
SMR	73	58	77	88	121	100						
Prostate (177)												
Observed	65	28	19	5	11	2						
SMR	72	59	80	77	115	69						
Bladder (181)												
Observed	35	24	5	1	3	2	14	6	2	5	1	—
SMR	86	93	56	59	81	286	92	63	57	833	83	—
Leukemia (204)												
Observed	23	12	8	2	1	—	15	7	2	3	—	3
SMR	88	88	114	125	31	—	93	74	53	333	—	750
Lymphomas (200–203, 205)												
Observed	40	23	9	4	4	—	25	20	2	—	3	—
SMR	147	149	134	308	129	—	152	208	48	—	200	—
All other sites												
Observed	98	53	21	7	12	5	62	41	11	1	8	1
SMR	111	108	94	143	136	167	97	113	67	30	133	50

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
	USSR											
All sites (140–205)												
Observed	1970	1169	370	116	248	67	1481	959	211	84	183	44
SMR	130	121	147	165	133	144	123	124	109	146	126	116
Buccal cavity and pharynx (140–148)												
Observed	25	11	2	4	7	1	8	4	—	1	1	2
SMR	39	26	21	138	99	50	67	51	—	167	77	333
Digestive system (150–159)												
Observed	969	586	194	48	110	31	694	466	96	36	73	23
SMR	152	145	179	154	147	173	158	163	137	159	149	174
Esophagus (150)												
Observed	61	38	12	2	7	2	42	34	4	—	1	3
SMR	158	143	185	143	219	222	442	515	308	—	100	1000
Stomach (151)												
Observed	353	202	75	21	45	10	182	117	22	14	24	5
SMR	187	178	227	198	179	161	210	217	159	269	224	161
Intestines (152–153)												
Observed	233	148	37	12	24	12	219	152	31	7	21	8
SMR	137	132	129	152	142	316	128	130	121	85	125	186
Rectum (154)												
Observed	121	77	28	7	8	1	74	56	5	5	7	1
SMR	119	108	188	184	76	67	120	132	56	200	106	83
Pancreas (157)												
Observed	108	67	19	5	13	4	71	43	15	2	9	2
SMR	140	145	145	132	115	160	163	161	214	91	143	143

Respiratory system (160–165)												
Observed	409	243	74	25	48	19	96	58	18	7	9	4
SMR	144	124	188	294	149	238	231	230	257	333	158	250
Lung and bronchus (162–163)												
Observed	380	223	72	23	44	18	90	55	16	7	8	4
SMR	155	132	212	311	154	269	249	251	267	389	157	286
Breast (170)												
Observed	2	2	—	—	—	—	198	127	26	12	28	5
SMR	57	83	—	—	—	—	78	75	68	113	94	86
Female genital organs (171–176)												
Observed							190	120	25	9	30	6
SMR							78	79	60	85	97	69
Cervix (171)												
Observed							37	22	4	3	7	1
SMR							50	50	30	97	66	29
Uterus, other and unspecified (172–174)												
Observed							49	30	6	3	8	2
SMR							57	56	38	71	86	63
Ovary (175)												
Observed							97	63	14	3	14	3
SMR							130	130	122	94	141	167
Male genital organs (177–179)												
Observed	115	59	21	12	19	4						
SMR	67	59	68	112	81	63						
Prostrate (177)												
Observed	111	57	20	11	19	4						
SMR	68	60	68	105	85	66						
Bladder (181)												
Observed	87	53	10	9	13	2	16	14	—	—	2	—
SMR	111	100	83	310	144	118	55	72	—	—	65	—
Leukemia (204)												
Observed	75	50	10	2	11	2	57	36	9	4	8	—
SMR	145	171	101	69	141	111	182	188	180	222	182	—
Lymphomas (200–203, 205)												
Observed	78	50	15	2	9	2	56	36	11	3	6	—
SMR	141	152	155	77	110	105	177	188	204	188	136	—
All other sites												
Observed	210	115	44	14	31	6	166	98	26	12	26	4
SMR	121	110	143	161	137	85	136	134	127	190	156	78

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
					Italy							
All sites (140–205)												
Observed	2671	1670	478	64	359	100	1384	901	244	42	143	54
SMR	103	94	120	127	125	130	85	80	94	141	89	121
Buccal cavity and pharynx (140–148)												
Observed	122	75	22	4	16	5	10	7	1	—	2	—
SMR	112	96	148	190	150	156	62	61	45	—	125	—
Digestive system (150–159)												
Observed	1335	808	236	34	203	54	632	417	111	18	60	26
SMR	124	109	138	154	176	182	107	100	120	165	110	166
Esophagus (150)												
Observed	126	74	15	1	33	3	9	6	1	—	1	1
SMR	189	152	142	100	660	214	73	65	63	—	91	333
Stomach (151)												
Observed	388	228	70	11	60	19	176	113	34	2	20	7
SMR	123	110	135	149	156	183	153	143	188	80	167	189
Intestines (152–153)												
Observed	314	188	55	11	49	11	166	115	27	9	12	3
SMR	109	91	121	200	189	172	72	68	80	220	64	58
Rectum (154)												
Observed	217	139	38	7	27	6	75	52	10	—	10	3
SMR	124	107	162	269	167	240	89	84	83	—	137	200
Pancreas (157)												
Observed	137	83	27	—	18	9	69	45	10	4	6	4
SMR	106	98	131	—	103	214	119	115	108	364	87	250

Respiratory system (160–165)												
Observed	450	297	82	8	51	12	60	39	9	2	8	2
SMR	91	83	129	121	101	94	108	106	99	182	125	105
Lung and bronchus (162–163)												
Observed	382	259	72	5	36	10	51	32	8	1	8	2
SMR	90	84	131	88	81	92	106	100	100	100	145	125
Breast (170)												
Observed	10	6	3	—	1	—	206	132	39	9	20	6
SMR	161	130	429	—	167	—	60	53	77	158	61	88
Female genital organs (171–176)												
Observed							250	155	49	5	31	10
SMR							76	69	87	86	92	98
Cervix (171)												
Observed							78	46	16	2	11	3
SMR							77	71	88	118	94	77
Uterus, other and unspecified (172–174)												
Observed							97	60	18	2	10	7
SMR							84	76	87	87	97	189
Ovary (175)												
Observed							65	43	13	1	8	—
SMR							64	60	83	53	73	—
Male genital organs (177–179)												
Observed	181	106	38	6	21	10						
SMR	63	57	79	86	59	93						
Prostate (177)												
Observed	173	101	37	5	20	10						
SMR	64	58	82	74	59	96						
Bladder (181)												
Observed	106	76	14	2	10	4	19	11	6	—	1	1
SMR	79	78	73	100	72	143	49	39	102	—	29	100
Leukemia (204)												
Observed	92	64	14	2	9	3	41	24	8	3	2	4
SMR	106	119	89	95	74	100	97	84	123	333	41	308
Lymphomas (200–203, 205)												
Observed	106	67	19	3	13	4	41	32	5	—	3	1
SMR	113	110	120	158	102	138	96	112	69	—	60	83
All other sites												
Observed	269	171	50	5	35	8	125	84	16	5	16	4
SMR	92	89	102	81	101	69	76	78	58	152	86	63

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
	Canada											
All sites (140–205)												
Observed	1052	340	348	71	241	52	1096	363	339	71	266	57
SMR	114	108	119	117	109	167	108	102	100	142	111	160
Buccal cavity and pharynx (140–148)												
Observed	52	17	26	1	7	1	15	4	6	—	5	—
SMR	145	124	248	42	85	91	153	111	200	—	217	—
Digestive system (150–159)												
Observed	482	171	156	27	105	23	438	150	144	29	93	22
SMR	125	130	124	100	118	193	114	111	110	137	110	173
Esophagus (150)												
Observed	30	8	10	2	9	1	13	3	1	2	7	—
SMR	147	96	143	182	26	200	181	100	45	1000	44	—
Stomach (151)												
Observed	167	58	51	7	46	5	107	37	42	6	18	4
SMR	138	156	130	74	150	116	130	139	151	120	93	129
Intestines (152–153)												
Observed	122	44	35	9	27	7	162	55	52	9	35	11
SMR	121	118	104	127	132	280	113	100	110	118	121	268
Rectum (154)												
Observed	65	24	24	4	10	3	58	23	15	3	13	4
SMR	115	102	145	121	81	33	114	114	93	136	116	36
Pancreas (157)												
Observed	48	21	15	2	6	4	46	18	10	3	12	3
SMR	104	144	103	69	47	267	119	144	79	143	115	250

Respiratory system (160–165)												
Observed	150	43	41	10	47	9	27	6	11	2	5	3
SMR	108	75	106	179	142	196	75	50	94	111	53	214
Lung and bronchus (162–163)												
Observed	130	39	33	8	43	7	26	6	10	2	5	3
SMR	109	80	101	182	147	184	84	59	98	133	62	300
Breast (170)												
Observed	5	3	1	—	1	—	210	63	65	13	63	6
SMR	313	429	200	—	333	—	105	82	104	153	136	115
Female genital organs (171–176)												
Observed							204	77	53	11	49	14
SMR							103	114	79	131	103	182
Cervix (171)												
Observed							73	34	19	3	13	4
SMR							116	173	90	136	78	129
Uterus, other and unspecified (172–174)												
Observed							63	22	17	4	16	4
SMR							89	93	65	114	111	133
Ovary (175)												
Observed							60	17	16	3	17	7
SMR							107	82	92	143	119	500
Male genital organs (177–179)												
Observed	149	41	50	15	36	7						
SMR	113	106	114	128	110	143						
Prostate (177)												
Observed	144	39	48	14	36	7						
SMR	115	108	115	124	116	146						
Bladder (181)												
Observed	44	14	17	4	8	1	26	9	6	2	6	3
SMR	96	80	120	182	73	100	100	95	70	154	103	429
Leukemia (204												
Observed	32	9	13	3	6	1	32	12	5	2	11	2
SMR	94	92	117	136	60	91	113	126	58	125	147	200
Lymphomas (200–203, 205)												
Observed	37	17	7	5	6	2	38	8	14	3	10	3
SMR	109	156	67	278	63	182	140	91	161	200	141	300
All other sites												
Observed	101	25	37	6	25	8	106	29	35	9	24	9
SMR	92	73	103	82	91	170	97	87	96	158	85	180

See footnotes at end of table.

APPENDIX A.—Standardized cancer-mortality ratios* (SMR = 100 for native-born whites) with number of deaths observed for foreign-born whites for selected primary sites by region† and sex, United States (35 States), 1950—Continued

Site (International List Numbers, sixth revision, 1948)	Males						Females					
	Total‡	North-east	East North Central	West North Central	West	South	Total‡	North-east	East North Central	West North Central	West	South
	Mexico											
All sites (140–205)												
Observed	218	6	14	15	179	4	254	2	14	9	226	3
SMR	91	115	65	122	92	89	122	56	104	106	127	70
Buccal cavity and pharynx (140–148)												
Observed	3	—	—	—	3	—	1	—	—	—	1	—
SMR	34	—	—	—	41	—	63	—	—	—	63	—
Digestive system (150–159)												
Observed	105	5	3	4	92	1	103	—	8	2	91	2
SMR	115	227	35	75	124	63	156	—	200	71	160	143
Esophagus (150)												
Observed	6	—	—	—	6	—	1	—	—	—	1	—
SMR	146	—	—	—	188	—	91	—	—	—	91	—
Stomach (151)												
Observed	52	2	3	3	44	—	34	—	5	2	26	1
SMR	177	400	125	188	181	—	238	—	625	286	210	333
Intestines (152–153)												
Observed	12	—	—	—	12	—	14	—	1	—	12	1
SMR	58	—	—	—	72	—	60	—	67	—	60	200
Rectum (154)												
Observed	6	2	—	—	4	—	12	—	1	—	11	—
SMR	48	667	—	—	38	—	136	—	200	—	139	—
Pancreas (157)												
Observed	13	1	—	1	11	—	13	—	1	—	12	—
SMR	96	500	—	143	97	—	169	—	250	—	174	—
Respiratory system (160–165)												
Observed	52	—	7	8	36	1	20	—	2	1	17	—
SMR	117	—	159	400	100	125	260	—	500	333	246	—

Lung and bronchus (162–163)												
Observed	48	—	7	8	32	1	20	—	2	1	17	—
SMR	122	—	184	471	100	167	313	—	667	500	288	—
Breast (170)												
Observed	—	—	—	—	—	—	20	—	—	—	20	—
SMR	—	—	—	—	—	—	45	—	—	—	53	—
Female genital organs (171–176)												
Observed							69	—	3	4	62	—
SMR							150	—	91	211	159	—
Cervix (171)												
Observed							41	—	3	1	37	—
SMR							241	—	214	167	257	—
Uterus, other and unspecified (172–174)												
Observed							16	—	—	3	13	—
SMR							120	—	—	600	117	—
Ovary (175)												
Observed							11	—	—	—	11	—
SMR							76	—	—	—	87	—
Male genital organs (177–179)												
Observed	19	—	2	1	15	1						
SMR	77	—	125	77	71	167						
Prostate (177)												
Observed	18	—	2	1	14	1						
SMR	80	—	143	83	74	167						
Bladder (181)												
Observed	11	—	—	—	10	1	3	—	—	—	3	—
SMR	111	—	—	—	118	—	77	—	—	—	83	—
Leukemia (204)												
Observed	5	1	—	1	3	—	3	1	1	—	1	—
SMR	45	500	—	200	32	—	47	—	250	—	18	—
Lymphomas (200–203, 205)												
Observed	3	—	1	—	2	—	6	1	—	—	5	—
SMR	23	—	83	—	19	—	95	—	—	—	89	—
All other sites												
Observed	20	—	1	1	18	—	29	—	—	2	26	1
SMR	59	—	30	59	66	—	122	—	—	200	125	250

*The standardized mortality ratios are expressed after adjustment for age by the indirect method, in terms of the corresponding cancer death rates for native-born whites residing in the same region.

†For regional grouping of States, *see* text-figure 1.

‡The SMR's for the total 35 States are based on the summation of observed and expected numbers of deaths in each region and are thus adjusted for the regional distributions of the foreign-born groups. These SMR's incorporating a regional adjustment are the ones presented in table 3.

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An Intermittent Perfusion and Gas Exchange Method for Continuous Cultivation of Cells in the Rose Chamber [1]

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SUMMARY

A simple technique for providing gas exchange within the Rose tissue-culture chamber is described. The method involves initial introduction of a large gas phase within the chamber, and subsequent maintenance of communication between this gas phase and a gas reservoir by means of polyethylene tubing. The fluid phase is exchanged intermittently by means of a gravity flow system through polyethylene tubes. The gas bubble within the chamber is kept in constant position, away from the microscopic field. We accomplish this by placing the entire apparatus at a slight angle to the horizontal, which creates a spirit-level effect. With this technique it is possible to grow and maintain heavy populations of strains P388 D_1, and HeLa cells for periods of 4 and 5 months, respectively. After these intervals the condition of the cells was as good as at the outset and was comparable to that of the same strain carried in tube cultures. There is no apparent time limit for growing cell strains within such chambers. Preliminary trials demonstrated that in this system submandibular gland rudiments of mouse embryos undergo development comparable to that in tube or dish cultures. The advantages of the system are that it permits long-term, time-lapse cinematography, with phase or ordinary optics, at any magnification, of cultures under conditions approximating those in standard tubes or flasks. The technique may be applicable to studies of long-term phenomena such as "spontaneous" or induced morphologic transformation of cells *in vitro*.—J. Nat. Cancer Inst. 26: 133–149, 1961.

MOST STANDARD tissue-culture vessels such as the roller tube, Carrel flask, T flask, Leighton tube, and Blake bottle provide conditions in which the volume of the gas phase is large in proportion to the volume of the fluid phase. In such standard closed vessels, the gas-volume : fluid-volume ratio usually varies from approximately 10:1 to approximately 20:1. Empirically, this relationship has been found satisfactory for the gas exchange requirements of established cell strains, under conditions varying widely as to the composition and frequency of replacement of both the gas and the liquid-phase components.

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Tissue-culture perfusion chambers used in time-lapse cinematographic studies are not designed to provide a similar volumetric gas: fluid proportion. Also, with the possible exception of the complex controlled gas-tension method described by Buchsbaum and Kuntz (*1*), tissue-culture chambers of the type used in phase-contrast cinematographic studies have not supported growth and maintenance of cell populations continuously in a manner that approximates the growth and maintenance of these same cell strains in tubes and flasks or other standard culture vessels. A chamber has been constructed by Spichtig (*2*) to provide continuous gas tension control, but actual results with cultures were not included in his report.

Chambers of the type used by Pomerat (*3*) have good optical qualities, but lack a gas-phase reserve. Hence, in order to meet the gas exchange requirements for continuous support of population densities comparable to those in standard vessels, such chambers must be provided with frequent exchange or continous flow of fluid medium greatly in excess of that required for nutrition alone. Excessive fluid medium supplies oxygen and removes carbon dioxide in solution at a rate sufficient to maintain cell respiration. Clearly, the conditions here differ radically from those in conventional closed culture vessels, in which relative fluid volume is small and fluid exchange is intermittent.

If the results of experiments performed in conventional vessels are to be repeated and recorded with the time-lapse cinematographic apparatus, it is essential that the conditions of the original experiments be duplicated as closely as possible in the perfusion chamber. Phenomena, such as "spontaneous" morphologic transformation *in vitro* and carcinogen- or virus-induced transformations, could be investigated by this means, if suitable long-term culture systems with good optical properties were available.

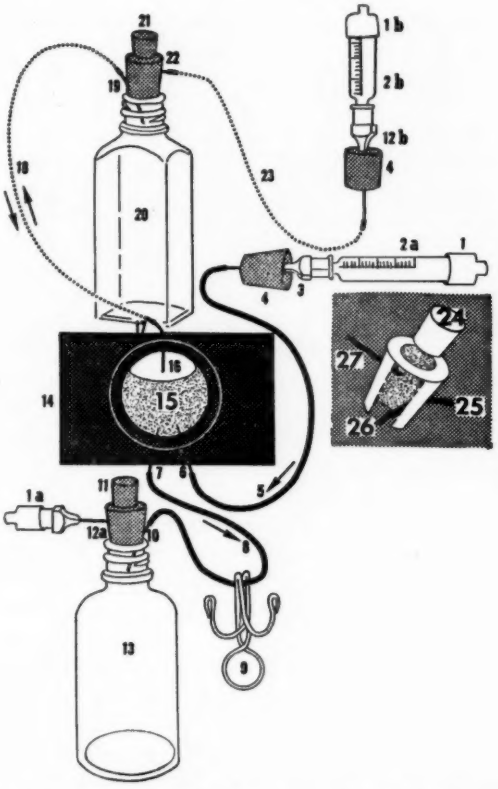
The purpose of this report is to describe a simple, inexpensive method of providing exchange of gas within a Rose chamber (*4*, *5*). With this method, cell strains HeLa (*6*) and P388 D_1 (*7*) were cultivated continuously under intermittent perfusion, and were observed with ordinary and phase-contrast optics concurrently with time-lapse cinematographic recording. Development of submandibular gland rudiments from embryonic mice has also been recorded cinematographically with the help of this apparatus.

MATERIALS AND METHODS

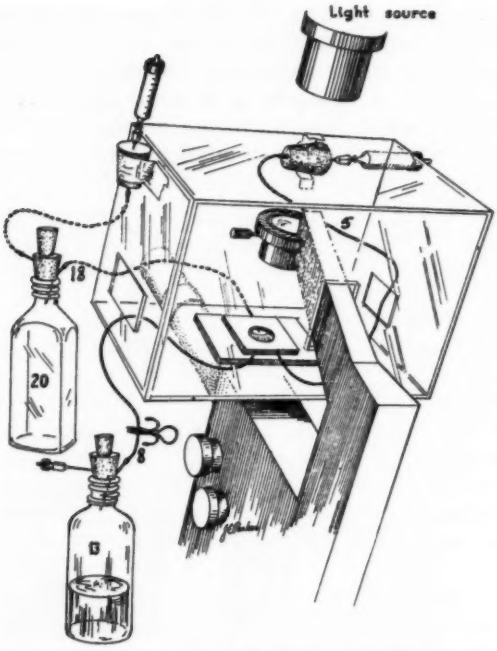
The Rose chamber.—The Rose chamber has been described elsewhere (*4*, *5*). The chambers used in this work were purchased from Wahlberg-McCreary, Inc., Houston, Texas, and were not structurally modified.

Description of parts of gas and fluid-exchange systems.—The apparatus is shown in text-figure 1. It consists of the Rose chamber with the following attachments: a fluid inflow tube (#5); a fluid outflow tube (#8);

a fluid receptacle with cap (#13 and 11); a gas exchange tube (#18); a gas reservoir (#20); and a gas reservoir cap (#21). These parts are attached to the chamber in a standardized fashion and are composed of a few simple components available in most tissue-culture laboratories. A diagram of the apparatus as used in conjunction with the time-lapse motion picture apparatus is shown in text-figure 2.



TEXT-FIGURE 1.—Diagram of Rose chamber together with attachments for gas and fluid exchange: 1, 1a, and 1b—vaccine vial caps; 2a and 2b—plugs (1 ml syringe tips); 3—20-gauge needle, 1½ inch; 4—white rubber stopper; 5—fluid inflow tube (polyethylene, PE 100); 6—20-gauge needle shaft (fluid inflow); 7—20-gauge needle shaft (fluid outflow); 8—fluid outflow tube (polyethylene, PE 100); 9—clamp (Day, pinchcock); 10—20-gauge needle shaft, 1½ inch (fluid receptacle entrance); 11—fluid receptacle cap (*see inset*); 12a and 12b—gas vent needle (18-gauge); 13—fluid receptacle; 14—Rose chamber; 15—fluid phase in chamber; 16—gas phase in chamber; 17—gas vent, (1½ inch, 18-gauge needle shaft); 18—gas exchange tube (polyethylene, PE 160); 19—18-gauge needle shaft, 1½ inch (gas reservoir entrance); 20—gas reservoir (60 ml); 21—gas reservoir cap (*see inset*); 22—gas vent needle shaft, 1 inch (18 gauge); 23—gas vent tube (polyethylene, PE 160). *Inset:* 24—white rubber stopper, #000; 25—white rubber stopper, S-43, with an 8 mm hole; 26—entrance needle, #10 or 19; 27—vent needle, #12a or 22.



TEXT-FIGURE 2.—Diagram of apparatus in use. Numbers refer to parts listed in text-figure 1. The gas reservoir (#20) and fluid receptacle (#13) are shown larger than scale. In actual use, the gas reservoir (#20) is taped to the side of the Lucite temperature-control box, and the fluid receptacle (#13) is held by a clamp and ringstand. The angle of tilt of the optical system is exaggerated here. The camera is below the field shown.

Fluid inflow tube.—Polyethylene tubing, 0.034 inch inside diameter (#5), is used for the introduction of nutrients and other fluids. At the distal, or upstream, end of the tube a 1.5 inch, 20-gauge needle (#3) is inserted and held in place by a white rubber stopper (#4). The stopper, anchored with clamp or tape, holds the needle in a position convenient for introduction of a syringe. The needle adapter is flamed lightly before feeding or other perfusion, to maintain sterility. Between perfusions, the inflow is closed by a removable plug (#2a), consisting of a 1.0 ml syringe tip capped by a vaccine vial cap (#1). A 20-gauge needle shaft (#6), which penetrates the rubber gasket and enters the chamber, is inserted into the proximal, or downstream, end of the tube. The inflow needle enters the same side of the chamber as the outflow needle (#8).

Fluid outflow tube.—Polyethylene tubing used for the fluid outflow (#8) is the same calibre as that used for the inflow. It also fits on a 20-gauge needle shaft (#7), which penetrates into the chamber. A Day pinchcock (#9) is used to open and close the outflow tube for removal of fluids. At the distal end, a slightly bent 1.5 inch, 20-gauge needle shaft

(#10) is inserted into the outflow tube. This needle shaft penetrates the fluid receptacle cap (#11) and thus connects the outflow tube with the fluid receptacle (#13).

Fluid receptacle and cap.—A 100 ml bottle is a convenient receptacle for the waste perfusate. The size and shape are not critical, although the neck of the bottle must be of a size that will accommodate the fluid receptacle cap (#11).

The fluid receptacle cap (*see inset*, text-fig. 1) is an S–43 white rubber stopper with an 8 mm hole bored through its long axis. This cavity allows easy entrance of the outflow needle shaft, which is bent and directed downward into the neck of the receiving bottle. This position of the shaft assures the unimpeded flow of the perfusate into the receptacle and lessens the chance of fluids clogging the passageway of the cap. Gas pressures are equalized by the removal of the vaccine vial cap (#1a), which covers the gas vent needle (#12a), and thus allows the gas pressure inside the fluid receptacle to come to equilibrium with the atmospheric pressure. The receptacle cap is plugged at the top by a small, white, rubber stopper (#24, *see inset* of text-fig. 1). Components of the fluid outflow system differ from those of the gas exchange system only in the size of the polyethylene tubing used.

Gas exchange tube.—Polyethylene tubing, 0.045 inch inside diameter (#18), is used as a gas exchange tube. Through this tube gases move by diffusion, and also probably by convection currents, between the gas reservoir (#20) and the gas phase within the chamber (#16). Theoretically, a larger calibre tubing would be more effective, but it was found by trial and error that PE 160, which fits tightly over an 18-gauge needle, is adequate. Studies have not been undertaken to determine the rates of gas movement or the precise upper and lower limits of the diameter and length of this gas exchange tube. The length of the tube in the present work was 40 cm.

In the proximal end of the tube is inserted an 18-gauge needle shaft (#17), which penetrates the rubber gasket and extends approximately 4.0 mm into the gas phase within the chamber. The 4.0 mm projection of the needle into the gas phase within the chamber prevents fluids at the edge of the gasket from being drawn into the gas exchange tube by capillary action. The needle is bent upward so that the end which fits into the gas exchange tube lies at an angle of approximately 60° to the surface of the chamber. This bend in the needle is used simply to avoid kinking and consequent obstruction of the polyethylene tubing. This tube must be kept absolutely free of fluids or other obstruction. Obstruction of the flow of gases for extended periods (24 to 72 hours) results in damage to the entire culture and death of a large percentage of the cells. Distally the gas exchange tube enters the gas reservoir cap (#21) and is extended into the gas reservoir (#20) in the same manner that was described for the fluid outflow tube and its accessories.

Gas reservoir and gas reservoir cap.—The gas reservoir is a glass container connected with the gas-occupied portion of the chamber by the gas ex-

change tube and gas reservoir cap just described. The optimal capacity of the reservoir has not been determined. However, we have observed that the tube alone will not permit sufficient gas exchange to sustain cell life in the chamber for an indefinite period. In repeated trials, clamping off the gas exchange tube resulted in death of all cells within 1 week. Furthermore, it was not possible to operate the system simply by allowing the gas exchange tube to communicate with the room air. When this was done, carbon dioxide was rapidly lost and the *p*H of the fluid phase within the chamber rose to levels incompatible with cell survival.

In developing this system, we sought a method whereby the ratio of the fluid phase to the gas phase would approximate, or even exceed, the fluid and gas relationships that exist in tube and flask methods of culture. Therefore, the volume of the gas reservoir (60.0 ml) was made 50 times greater than the volume of the fluid phase (1.2 ml).

The gas vent (#22 and 23) is extended from the gas reservoir cap to put its needle entrance in a position where it can be flamed readily, away from the Lucite incubator. We maintain sterility by flaming the needle entrance lightly when the plug is removed and replaced.

Sterilization.—Rubber and glass components of the apparatus are boiled in alkaline detergent (Calgolac), and rinsed with tap and distilled water. They are then autoclaved for 30 minutes at 18 pounds pressure. Coverslips are cleaned in 95 percent ethanol and autoclaved for 30 minutes at 18 pounds pressure. Metal plates and screws are rinsed in 95 percent ethanol and sterilized in the autoclave. The latter parts do not come in contact with the culture fluids, but we have found it convenient to sterilize all parts of the chamber and keep them together in a sterile petri dish while assembling them with a sterile forceps. The possibilities for contamination are thus minimized. We sterilize the polyethylene tubing [3] by flushing a mixture of equal parts of ethyl ether and 95 percent ethanol through the tubing for 5 to 10 minutes. Polyethylene is soluble in this mixture and longer contact with it is damaging. To remove the alcohol-ether mixture, the tubing is rinsed with sterile saline or nutrient medium. It is necessary to dry only the gas exchange tube: A sterile needle and syringe are attached and air is forced through the tubing. When these steps have been taken, the apparatus is ready for assembly and subsequent injection of a cell suspension or introduction of explants in the manner described by Rose (*4*).

Assembly and culturing.—The chamber is assembled in the manner described by Rose (*4*). Prior to sterilization, the needles and needle shafts are placed into the rubber components as they appear in text-figure 1. This is done before sterilization and assembly of the chamber to allow removal of small cores of rubber that become lodged in the lumens of the needles in the process of insertion. Otherwise, these rubber cores are expelled into the chamber during fluid injections. Once in place, the needles need not be removed for repeated washing or sterilization.

[3] This tubing can be purchased in sterile condition from Clay-Adams, Inc., 141 West 25th St., New York 10, N.Y.

The presence of the needles within the rubber gasket during autoclaving does not affect the tightness of fit or result in damage to the rubber. After sterilization the rubber, glass, and metal components are arranged as shown in text-figure 1. When the polyethylene tubing has been attached, the assembled apparatus is ready to receive tissue explants or cell suspensions.

To seed the chamber, we first remove the syringe-tip plugs, and the needles from which they were removed are gently flamed with a small gas flame. A cell suspension is then injected into the chamber through the fluid inflow tube. During this procedure, the gas exchange tube functions as an escape route for air displaced by the incoming fluid suspension. When the fluid reaches approximately two thirds of the capacity of the chamber, the syringe is withdrawn. The two syringe-tip plugs are replaced after the needles into which they fit have been flamed.

The chamber should at no time be filled to capacity with fluid. This is necessary to avoid fluid obstruction of the gas exchange tube, which must remain completely open if it is to function. It was found that 1.2 ml, equivalent to two thirds of the chamber's capacity, is a workable volume for the fluid phase (#15).

To maintain the gas phase in the chamber in a constant position in contact with the lumen of the gas exchange needle, it is necessary to tilt the microscope, camera, and light source. We easily accomplished this with our apparatus, shown in text-figure 2, by tilting to an angle of 8 to 10° the cast-iron table on which all parts of the apparatus are mounted in optical alignment. Tilting at this angle also helps to clear the microscopic field of cell debris, because much of this material gravitates to the lower side of the chamber and is siphoned off through the fluid outflow tube at feeding time.

The feeding procedure is as follows: A 2.0 ml syringe is filled with nutrient medium. The vaccine vial cap and the syringe-tip plug (#1a and 2b) are now removed from the two gas vent needles (#12a and 12b). The adapters of these needles are sterilized with a small gas flame. Then the clamp is removed from the fluid outflow tube. The perfusate, along with any loose cell debris, flows off by gravity into the fluid receptacle, as the latter is arranged lower than the chamber. When the desired volume of perfusate has been drained off, we stop the flow by replacing the clamp on the outflow tube. After this procedure, the desired quantity of fresh nutrient is injected slowly into the chamber through the fluid inflow needle (#3). The vaccine vial cap and the syringe-tip plug (#1a and 2b) are returned to their respective positions after the corresponding gas vent needles are flamed. The feeding procedure is thus completed in not more than 5 minutes.

OBSERVATIONS ON PERFORMANCE

Two tissue-culture cell lines were cultivated and photographed during long periods within this chamber (figs. 1 to 12). Strain HeLa (*6*) was

grown continuously for 5 months, and a polyoma virus-carrier substrain of P388 D_1 cells (*7*, *8*), derived from a murine lymphoma, was grown continuously for 4 months. Both these cell lines currently are being carried continuously in the chamber, and there appears to be no theoretical or practical reason why it should not be possible to carry them indefinitely in this fashion. Bacterial contamination has occurred only once, immediately after assembly of the apparatus and seeding of the cells. Thus far, after hundreds of fluid exchanges in several chambers, there has been no instance of contamination that could be related to the feeding procedure.

In preliminary experiments with salivary-gland rudiments from 13- and 14-day mouse embryos, time-lapse pictures of single rudiments on the surface of a thin plasma clot were taken for 36 hours (figs. 13 to 18). One such culture was maintained for 30 days before fixation, with results comparable to those seen in tube cultures. Development of branching epithelial tubules from the single-bud stage was thus recorded, and permitted observations on the dynamic aspects of the process.

The characteristics of cell distribution within the chamber are of interest and also important with respect to practical applications. With both the HeLa and the P388 D_1 cultures, the cells did not remain uniformly distributed over the portion of the glass surface covered by the fluid phase. Rather, they mainly aggregated close to the liquid-gas interface, though they also occupied adjacent areas extending below the fluid surface as well as an area well above the visually apparent meniscus of the fluid level. After a month in the chamber, HeLa cells differed from P388 D_1 cells in distribution, in that they grew in multilayered clusters far above the apparent meniscus of the fluid and covered the glass in an area that at first appeared to be entirely above the fluid phase. Careful inspection of the chamber in position on the stage revealed, of course, that these cells were covered by a thin film of fluid making up the portion of the meniscus that was greatly extended as a result of the tangential relationship of the fluid surface to the glass surface. Apparently only an extremely thin film of nutrient fluid over HeLa cells was sufficient to support survival and growth under the conditions prevailing in the chamber. Later on, however, after the same culture had been carried for 5 months, the HeLa cells no longer grew far above the meniscus, but populated the entire area below it. Processes of selection or adaptation might account for such a change.

P388 D_1 cells, on the other hand, at no time distributed themselves far up along the tangential portion of the meniscus, but covered the glass to a greater depth below the visually apparent, or perpendicular, portion of the meniscus. Viable cells of this strain were found in abundance as far as 1.6 cm below the lowest point of the liquid-gas interface. These differences of distribution between HeLa cells and P388 D_1 cells, with the liquid-gas interface as a reference point, would appear to reflect differences in the optimal gas tension requirements of these two cell lines. This observation is of practical importance to phase-optics photography, because satisfactory phase contrast can be obtained only at levels where the light beam

does not pass through a liquid-gas interface within the chamber. Phase-contrast microscopy of both HeLa and P388 D_1 cells was readily possible, but the zone populated by HeLa cells under favorable phase-optics conditions was narrower than that populated by P388 D_1 cells.

Cell proliferation rates have not been measured in these chambers by cell-count methods, as studies of growth rates *per se* were not an objective in the development of the apparatus. At the present it can only be stated that after the chambers were seeded with a small cell population (in the order of 1,000 cells), the population increased rapidly during a 2-week period. During the following 2 weeks the cells gradually redistributed themselves until a heavy population zone was established as described previously. Thereafter, cell death and cell replacement reached a state of equilibrium that remained approximately constant for a fixed feeding schedule. The population density in some areas was greatly in excess of that desired for single-cell observations, so that a problem of restricting population density arose. A fully satisfactory solution to this problem has not been obtained, but we are able to remove HeLa cells from the chamber without moving the chamber from the field by running a 5 percent trypsin solution through the fluid exchange system.

One of the objectives in the development of this apparatus was to make possible continuous observation of the effects of polyoma virus on P388 D_1 cells. The development of full-blown cytopathic changes in this cell-virus system had previously required a minimum of 7 days, when carried out in Leighton tubes or 2-ounce medicine bottles. Preliminary trials have been made with the same cell-virus system in the apparatus described here. Development of a complete cytopathic effect was observed in only a little more than 4 days, under the same feeding schedule and gas exchange conditions that allowed uninfected P388 D_1 cells to maintain themselves in a flourishing population for at least 4 months, and presumably for an indefinite period. Time-lapse photography of the process was carried out with ordinary optics during one of these preliminary trials (figs. 7 to 12). The observations will not be elaborated in this technical report, but it is relevant to note that the shortened time required for development of the cytopathic effect in the chamber suggests that the conditions provided by it were not identical with those prevailing in the culture vessels previously used.

DISCUSSION

The intermittent perfusion and gas exchange culture method described in this report permits continuous cultivation of at least two established cell strains in the Rose chamber. It does not solve all the problems confronting investigators whose objectives are to observe directly and record photographically long-term processes in cell, tissue, or organ cultures. Realizing that this technique constitutes only a step toward these ideal conditions, we will outline its advantages and disadvantages as clearly as we have been able to recognize them.

The advantages are:

1) The apparatus and technique allow continuous observation at all ranges of light-microscopic magnification, over periods of time theoretically unlimited.

2) The chamber and the microscopic field need not be moved at any time for purposes of fluid or gas exchange. Within limitations, cells or small tissue pieces can be added or removed without the removal of the chamber, the microscopic field, or the microscopic focus.

3) Conversely, if desired, the chamber and its attachments can be removed from the incubator box of the time-lapse cinematographic apparatus, and the culture can be continued in any ordinary incubator, for subsequent return to the photographic system whenever convenient. Thus several chamber cultures can be maintained in an incubator or warm room for periodic photographic observation as required. In certain types of study, *e.g.*, observation of cell motility, internal cell structure, nuclear rotation, or pinocytosis, this feature eliminates the task of repeated preparation of new chamber cultures for each series of observations.

4) Observations made with this system are more nearly comparable to those made in ordinary culture vessels than are those made with perfusion chambers not incorporating gas exchange features. This point has already been elaborated earlier in this report. Because of this feature, it should be possible to study long-term tissue-culture phenomena previously observed without benefit of continuous time-lapse photographic recording, *e.g.*, morphologic transformations of "spontaneous", viral-induced, or chemical-induced origin.

5) The technical assembly and operation of the apparatus are simple and can be accomplished in any laboratory with the most elementary tissue-culture methods. The inherent advantages of the Rose chamber are preserved without addition of complicated instrumentation or precision-built parts. Problems of leakage, breakage, and contamination are practically nil.

Disadvantages of the method are:

1) The gas exchange in the system as described here is without close control. As with gas exchange in standard closed-vessel cultures, the prevailing gas tensions at any given time are unknown. It is only known that the conditions provided support cell survival and proliferation. Many factors influence the gas exchange, such as the frequency of fluid exchange (a procedure which concomitantly draws air into the gas reservoir), frequency of opening the gas reservoir, and fluctuations of temperature in the incubator chamber, which result in gas volume fluctuation and consequent fluctuation in direction and amount of gas movement in the gas exchange tube. Other factors are the size of the gas bubble within the chamber, the length and diameter of the gas exchange tube, the volume of the gas reservoir, and the room temperature which governs the temperature of the gas reservoir. Fortunately, most of these variables can be kept relatively well controlled.

2) The chamber, and consequently the entire optical system, must be kept at a slight angle to the horizontal. Some time-lapse cinematographic equipment may not lend itself readily to this modification. Two possible ways of modifying the apparatus to eliminate this disadvantage have occurred to us: (*a*) to incorporate a gas-containing antechamber, or well, on the upper plate of the Rose chamber and to the side of the viewing aperture. The gas exchange tube would then enter this portion of the chamber, and gas exchange could take place at the broad interface with the fluid medium beneath; (*b*) to confine the gas phase within a permeable membrane such as cellophane or plastic, which would be attached to the gas exchange needle and would occupy the same space as the gas bubble in the chamber described in this report.

3) The Rose chamber, with a gasket of standard 3.5 mm thickness, is too deep to permit phase microscopy with a standard phase condenser. The availability of long working-distance phase condensers partially obviates this disadvantage. Such a condenser was used in this work.

Gas tensions at various places within the culture chamber are probably not uniform. Evidence of this is the zonal distribution of cells in a pattern roughly paralleling the liquid-gas interface. This is a serious objection because it means that one's observations theoretically could be influenced, depending on which area of the chamber is selected for study. It does not negate the usefulness of the chamber, for the same objection applies to most other stationary culture vessels. A remedy such as a to-and-fro motion of the fluid in the fluid inflow and outflow tubes is conceivable.

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PLATES

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PLATE 5

FIGURES 1, 2, and 3.—Three consecutive frames from a time-lapse motion picture film, showing a strain HeLa cell in a culture that had been maintained in a Rose chamber with gas exchange attachments for 4 months. Dark phase contrast. × 900

FIGURES 4, 5, and 6.—Three consecutive frames from a time-lapse series of another HeLa strain cell of same culture illustrated in figures 1, 2, and 3. Dark phase contrast. × 900

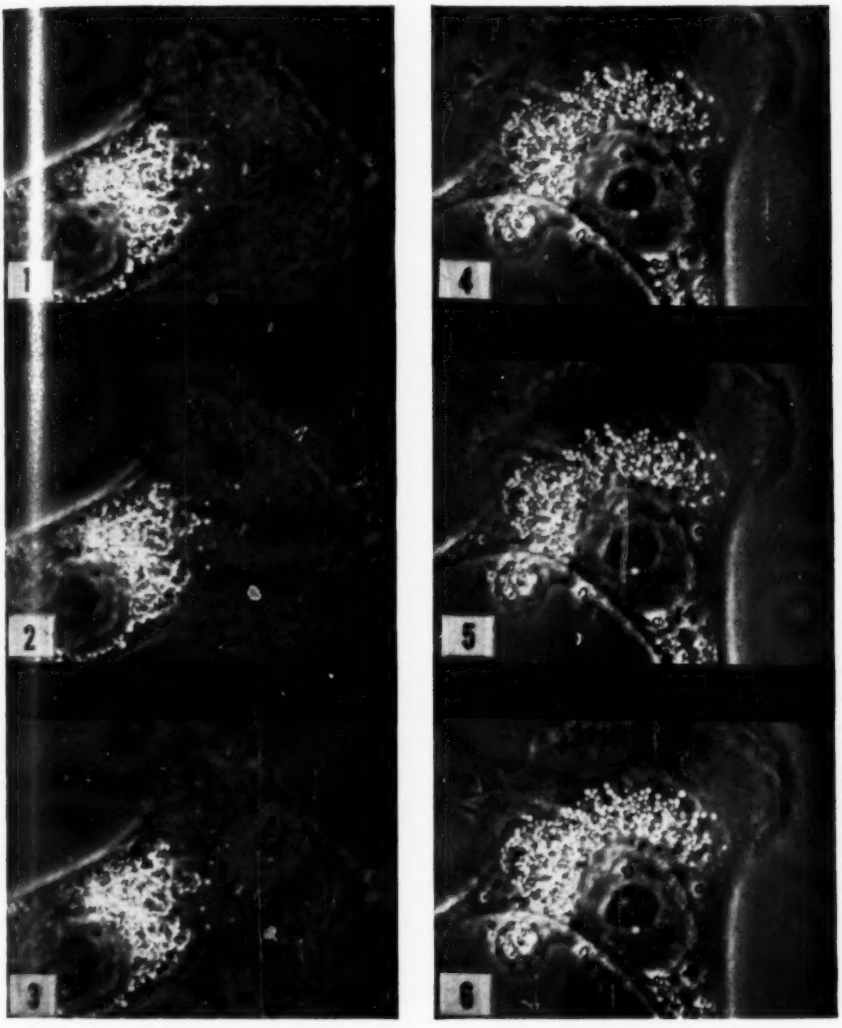
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PLATE 6

FIGURES 7 and 8.—Two consecutive frames from a time-lapse photographic series of strain P388 D_1 culture in a Rose chamber with gas exchange attachments. Cells had been seeded into chamber on preceding day and had been infected with polyoma virus 4 hours before photographing. No effects of virus are evident at this time. Photographed without phase optics. × 60

FIGURES 9 and 10.—Two consecutive frames from same series illustrated above, 48 hours after infection with polyoma virus. Cells show granule accumulation in cytoplasm and tendency to clump characteristic of early cytopathic response of these cells to polyoma virus when grown in high-serum medium. Photographed without phase optics. × 60

FIGURES 11 and 12.—Two consecutive frames from same series illustrated in figures 7 to 10, 96 hours after infection with polyoma virus. Clumping is marked and most cells are dead and disintegrating. Cytopathic effect is nearly complete. Photographed without phase optics. × 60

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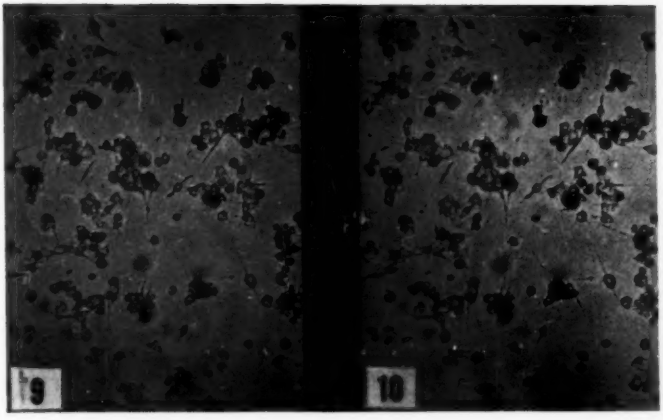
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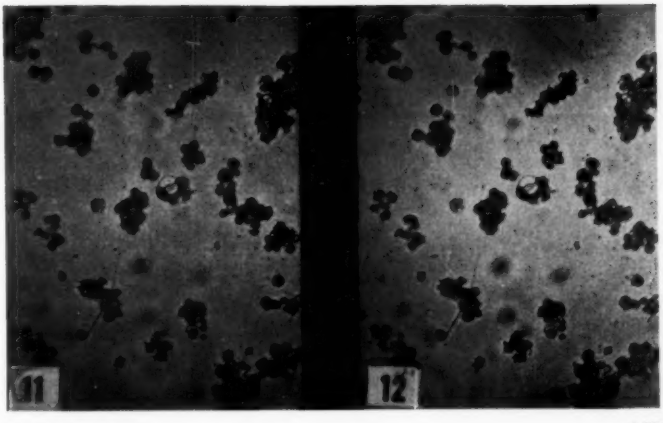
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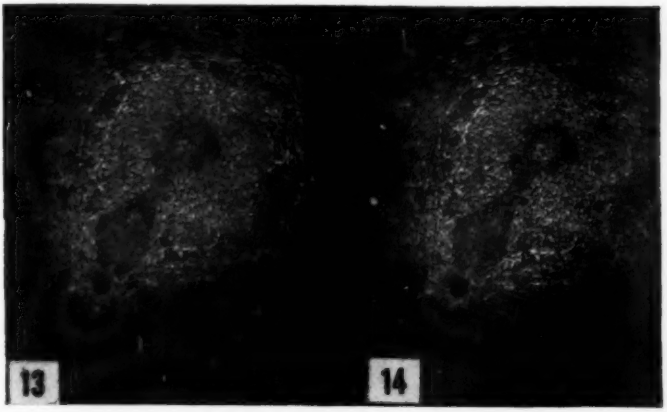
PLATE 7

FIGURES 13 and 14.—Two consecutive frames from a time-lapse photographic series of a mouse salivary-gland rudiment undergoing morphogenesis in Rose chamber with gas exchange attachments. Rudiment was from a 13-day C3H/Bi embryo, and had been in culture 12 hours at time of this photograph. Dark phase contrast. × 30

FIGURES 15 and 16.—Two consecutive frames from same series shown in figures 13 and 14, but 14 hours later. *Note* appearance of buds at periphery of terminal portion of epithelial component. Dark phase contrast. × 30

FIGURES 17 and 18.—Two consecutive frames from same series shown in figures 13 and 14, 36 hours later. *Note* well-formed branches from epithelial component, and cavitation of duct. Dark phase contrast. × 30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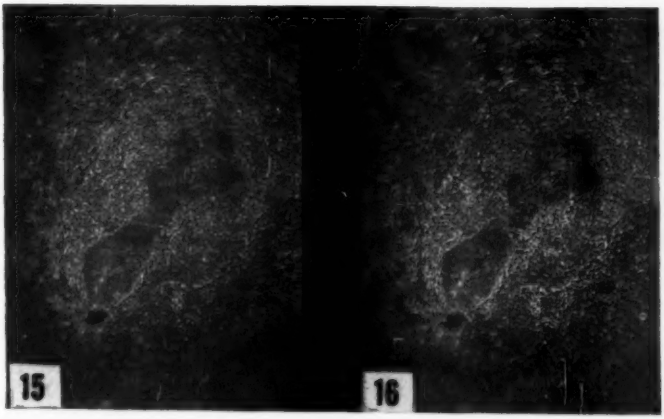
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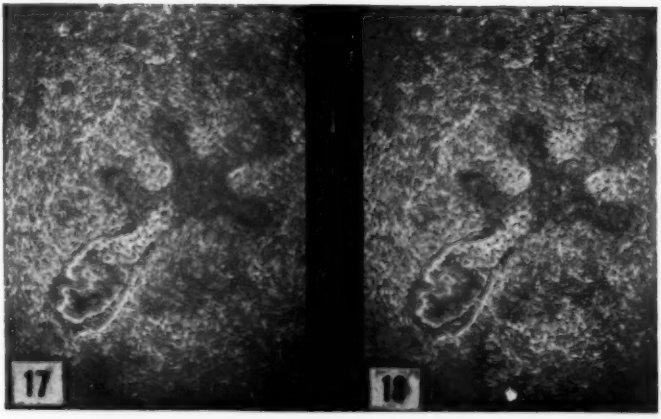
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Sex Difference in Liver Tumorigenesis in Rats Ingesting *N*-2-Fluorenylacetamide [1]

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SUMMARY

The sex difference in liver tumorigenesis in Buffalo-strain rats ingesting *N*-2-fluorenylacetamide (2-FAA) was investigated. In one experiment male and female rats were fed a diet containing 0.03 percent 2-FAA. Male rats had a higher incidence of liver tumors (12 of 12) than the female rats (5 of 14). In a second experiment female rats fed a diet containing 0.06 percent 2-FAA also had a low incidence of liver tumors (6 of 15). In a third experiment, in which the average duration of survival of female rats fed a diet containing 0.03 percent 2-FAA was prolonged by surgical removal of breast tumors, a higher incidence of liver tumors (7 of 9) was found. Morphologically the liver tumors in male rats were less differentiated and more anaplastic than those of female rats. Histologic studies of the livers of both sexes ingesting a diet containing 0.03 percent 2-FAA and killed at frequent intervals for 4 months demonstrated that certain histologic changes in the livers of male rats were present earlier and were different from those in the livers of females. Testosterone administration influenced the development of the early histologic changes in the livers of male rats. Detailed histologic findings are presented—J. Nat. Cancer Inst. 26: 151–187, 1961.

DURING A study on the influence of dietary intake of pyridoxine on tumorigenesis in rats ingesting *N*-2-fluorenylacetamide (2-FAA) (*1*), liver tumors from a number of rats of both sexes were examined. Tumors developed more frequently in male than in female rats of the Buffalo strain (*2*) that ingested 2-FAA in diets containing adequate or elevated amounts of pyridoxine. Histologic examination showed certain morphologic differences in the liver tumors of the two sexes.

These findings led us to investigate a few factors that might explain this difference. We conducted two experiments to learn whether an increase of either the length of survival or the level of carcinogen intake of female rats would alter the incidence of liver tumors. In one chronic experiment the average survival of female rats ingesting 0.03 percent 2-FAA was prolonged by the surgical removal of breast tumors when they developed. In the other experiment female rats were fed a diet

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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containing 0.06 instead of 0.03 percent 2-FAA. These chronic experiments indicated that the incidence of liver tumors in female rats ingesting 0.03 percent 2-FAA increased as the rats survived longer, but did not increase with a higher level of carcinogen intake.

Morphologically our results indicated that the liver tumors of the male rats were less differentiated and more anaplastic than those of the female rats. Because of these findings we became interested in the sequence of histologic changes in the livers of male and female rats ingesting a diet containing 0.03 percent 2-FAA and killed at frequent intervals for 4 months. This study revealed that certain histologic changes in the livers of male rats were present earlier and were different from those in the livers of female rats. In a second acute experiment, the influence of testosterone administration on the development of the early histologic changes in the livers of male rats was investigated. Groups of intact females, intact and castrated males, with or without testosterone administration, were fed a diet containing 0.03 percent 2-FAA, and representative animals from each group were killed at 1, 2, and 4 months. The results indicated that testosterone influenced the early histologic changes in the livers of male rats.

This report is concerned with acute experiments in which the sequence of morphologic changes in the livers of male and female rats was studied, and with chronic experiments concerning the role of survival, the dosage of carcinogen on the sex difference in tumor incidence, and the morphologic differences of the tumor types.

MATERIALS AND METHODS

Inbred male and female rats of the Buffalo strain (*2*) were used. At the beginning of each experiment the animals varied from 2 to 2.9 months in age. The basal diet used in all except one experiment is summarized in table 1. Rats on the experimental diet were fed the basal diet containing 0.03 percent 2-FAA in all but one experiment—chronic experiment

TABLE 1.—Composition of basal diet

g/kg Diet	
Casein	260
Lard	150
Salt mixture	40
Cellulose flour	20
Dextrin	530
μg/g Diet	
Thiamine·HCl	4
Riboflavine	8
Pyridoxine·HCl	25
Niacin	4
Calcium pantothenate	20
Choline chloride	2000
Tocopherol	150
Cod-liver oil	4000

3, in which the basal diet was the same as described in table 1 except that it contained a different vitamin mixture supplying the following in μg per g diet: thiamine·HCl 8, riboflavine 16, pyridoxine·HCl 8, niacin 8, calcium pantothenate 40, choline chloride 2000, and cod-liver oil 4000. In this experiment rats on the experimental diet received the basal diet containing 0.06 percent 2-FAA.

All animals were housed individually in wire cages with raised bottoms. Diet and water were available at all times. Each animal was weighed, and the diet it consumed was measured at weekly intervals. Fresh food was given each week. The room temperature was maintained at 78 ± 1° F. In all chronic experiments animals on the experimental diet were studied until death or until they became moribund and were killed. At the beginning of the first chronic experiment all the animals were 2.3 months old. Control animals on the basal diet in this experiment were killed after an average of 9.4 months. In the second chronic experiment female rats were started at 2.4 and males at 2.5 months of age. Females were examined frequently for the development of mammary tumors, and when these were found, simple mastectomies were performed with ether anesthesia. All control animals on the basal diet were killed after 12.2 months. In the third chronic experiment female rats were started at 2.9 months of age, and the control animals were killed after 10.4 months.

In one acute experiment groups of male rats started at 2.7 months of age were killed at weekly intervals for 4 weeks and each month thereafter for 3 months. Female rats started at 2.8 months of age were killed at monthly intervals for 4 months. All animals were killed with gas. In another acute experiment the influence of testosterone on liver changes of rats ingesting a diet containing 0.03 percent 2-FAA was studied. Male and female rats 2 months old were divided into 7 groups as follows: 1, intact females; 2, intact females plus testosterone; 3, intact males; 4, intact males plus testosterone; 5, castrated males; 6, castrated males plus testosterone; and 7, castrated males plus testosterone (oral). Each animal in groups 2, 4, and 6 received subcutaneously 500 μg testosterone proprionate in sesame oil daily for 5 days, Monday through Friday of each week, throughout the experiment. Animals in group 7 received methyl testosterone in the diet (16.7 mg/kg). Representative animals from each group were killed with gas at 1, 2, and 4 months.

All the animals were autopsied, and the tissues were fixed in Zenker-formol solution. The liver of each animal was weighed, and many pieces were taken from each lobe. Other organs routinely taken in the chronic experiments were lung, kidney, thyroid, pituitary, adrenals, pancreas, bladder, and gonads, and additional material was taken from any area that appeared abnormal. In the acute experiments pieces of liver and of gonads were taken. All sections were routinely stained with hematoxylin and eosin, and selected tissues were also treated with mucicarmine stain, periodic acid-Schiff stain, acid-fast stain, Sudan IV stain, iron stain, Mallory's phosphotungstic acid-hematoxylin stain, Masson's trichrome stain, and van Gieson's stain.

RESULTS

Chronic Experiments

Experiment 1.—Male and female rats were fed the basal control diet and the experimental diet containing 0.03 percent 2-FAA. All 12 male rats and 5 of 14 females on the experimental diet developed liver tumors. No liver tumors were found in the 9 control animals, 3 males and 6 females, fed the basal diet. Table 2 summarizes the findings in this experiment.

Histologic examination of numerous sections of liver from each male and female rat revealed differences in the tumors of the two sexes. The liver tumors of males could readily be diagnosed as hepatocellular carcinomas, either trabecular or anaplastic. After careful examination of the livers of females, however, it was difficult in many instances to determine whether some lesions were neoplastic or hyperplastic. In questionable cases the lesions were considered as hyperplastic. The over-all impression was that the liver tumors in the males were less differentiated and more anaplastic carcinomas (figs. 1 and 2) than those in the females (fig. 3). These histologic findings suggested a higher degree of malignancy of the liver tumors in males than in females. This observation was supported by the finding of lung metastases from the liver in 8 of 12 males (figs. 4 and 5), but in none of 5 females that had liver tumors. No tumors or pathologic changes were observed in the livers of the control animals.

Detailed histologic examination of sections of livers from the 12 male rats ingesting 0.03 percent 2-FAA in the diet for an average of 6.7 months revealed that each liver contained multiple sites of carcinoma. According to the classification of Stewart and Snell (*4*) these tumors were trabecular (fig. 4) or anaplastic carcinomas (figs. 1 and 2). The trabecular carcinomas in the livers of these animals could be further subclassified according to Edwards and White (*5*) as hepatomas, type II. All livers in the males contained areas demonstrating trabecular carcinomas, and 6 had areas of anaplastic carcinoma. All livers had many cystic areas with cuboidal or flattened epithelium-like cell linings (fig. 6), small to large areas of necrosis, large hemorrhagic areas usually adjacent to tumor (fig. 7), frequent macrophages containing finely granular pigment, and multiple areas of nodular hyperplasia. In 7 of the livers there was a mild fatty change.

Five of the 14 female rats ingesting 0.03 percent 2-FAA for an average of 6.4 months had trabecular carcinomas (fig. 3), and 1 liver had a hemangioendothelioma. According to the classification of Edwards and White (*5*), the well-differentiated trabecular carcinomas in the livers of the females were hepatomas, type I. Two livers with trabecular carcinoma had, in addition, areas of a well-circumscribed angiomatous liver tumor which contained hemorrhagic and necrotic material in the center with tumor cells, resembling pleomorphic hepatic cells, at the periphery (figs. 8, 9, and 10). Some of these tumor cells were adjacent to blood spaces lined by endothelial cells. This tumor was different from the other liver tumors

Table 2.—Liver tumors in male and female rats ingesting 2-FAA in diet in chronic experiments

Experiment number	Group	Sex	Number of rats	Duration average in months	Diet intake		2-FAA intake		Liver weight		Liver tumors	Lung metastasis
					g/rat/day	g/rat/day/100 g body weight*	mg/rat/day	mg/rat/day/100 g body weight*	Gram	g/100 g body weight		
1	Control	M	3	9.0 ± 2.2†	10.3 ± 0.3†	3.1 ± 0.2†	0	0	8.5 ± 0.9†	2.8 ± 0.2†	0	0
	Control	F	6	9.8 ± 0.8	7.6 ± 0.2	3.3 ± 0.2	0	0	6.7 ± 0.9	3.2 ± 0.4	0	0
	0.03% 2-FAA	M	12	6.7 ± 0.2	8.4 ± 0.1	3.4 ± 0.1	2.5 ± 0†	1.0 ± 0†	21.6 ± 2.0	9.6 ± 1.0	12	8
	0.03% 2-FAA	F	14	6.4 ± 0.2	7.2 ± 0.2	3.7 ± 0.1	2.2 ± 0.1	1.1 ± 0	9.9 ± 1.0	5.7 ± 0.4	5	0
2	Control	M	2	12.2 ± 0	11.2 ± 0.1	3.0 ± 0.1	0	0	11.5 ± 0.1	2.8 ± 0.1	0	0
	Control	F	3	12.2 ± 0	9.3 ± 0.3	3.5 ± 0.1	0	0	10.0 ± 0.5	3.6 ± 0.7	0	0
	0.03% 2-FAA	M	6	6.3 ± 0.4	7.2 ± 0.3	3.2 ± 0	2.2 ± 0.1	1.0 ± 0	12.9 ± 1.9	6.5 ± 1.0	5	0
	0.03% 2-FAA	F	9	8.2 ± 0.4	6.8 ± 0.2	3.8 ± 0.1	2.1 ± 0	1.1 ± 0	12.1 ± 1.2	7.4 ± 0.6	7	4
3	Control	F	9	10.4 ± 0.9	8.1 ± 0.2	3.4 ± 0.1	0	0	8.2 ± 0.4	3.6 ± 0.4	0	0‡
	0.06% 2-FAA	F	15	6.7 ± 0.3	6.5 ± 0.3	4.1 ± 0.3	3.9 ± 0.2	2.5 ± 0.2	9.1 ± 0.7	6.5 ± 0.6	6	0‡

*Average diet or 2-FAA intake in g or mg/rat/day/100 g body weight. Body weight was determined from average weekly body weights.

†Mean ± standard error of the mean, calculated by the method of Mantel (5).

‡No gross evidence of lung metastases. No histologic sections of lung taken in experiment 3.

described earlier—we feel that it can best be classified as an angiomatous hepatocellular carcinoma. All 14 livers showed mild-to-moderate fatty change, 11 had multiple areas of nodular hyperplasia, 9 had pigment-laden macrophages, 7 had small areas of necrosis, 5 had cystic areas, and 4 had areas of hemorrhage.

To evaluate the differences between the two sexes in incidence and in histologic types of the liver tumors, we reviewed the dietary and 2-FAA intake (table 2) and the complete autopsy data of the rats. The dietary and carcinogen intake was somewhat different in the sexes, but when these values were corrected for average body weights, the difference was minimal. Both sexes survived approximately the same average length of time: females, 6.4 months, and males, 6.7 months. The autopsy protocols revealed that the majority (67%) of male, but no female, rats that died with liver carcinoma had lung metastases; however, most females (93%) had breast cancers which probably contributed to their deaths. No breast tumors were found in the males.

Experiment 2.—To prolong the survival time for the female animals ingesting a diet containing 0.03 percent 2-FAA, we performed a simple mastectomy as soon as a breast tumor was observed or palpated in animals of another experiment (table 2). Nine female rats were studied for an average of 8.2 months and 6 males for 6.3. Sixty-four adenocarcinomas and 4 sarcomas of the mammary glands were found in 8 of the 9 female animals. Two females that lived 6.1 and 6.4 months had no liver tumors. Seven females that lived from 7.3 to 9.9 months had liver tumors, 4 of which had lung metastases. Five of the 6 males developed liver cancers smaller in number and size than those found in experiment 1, as reflected by the difference in liver weights. No mammary tumors developed in the males. The histologic findings in the livers of both sexes were similar to those described earlier in experiment 1. Three of the 7 females that had trabecular carcinomas of the liver had, in addition, areas of an angiomatous hepatocellular carcinoma. Vascular invasion within the liver and also metastases to the lungs by nests of cells resembling the primary tumor were observed in these animals. Five control animals on the basal diet for an average of 12.2 months had no pathologic changes in the livers.

Experiment 3.—From results of the first 2 chronic experiments, it was apparent that when the animals ingested a diet containing 0.03 percent 2-FAA, male rats developed liver tumors earlier than females. For this reason we wished to determine whether or not the rate of tumor development or the over-all incidence of liver tumors in females could be increased by a higher intake of 2-FAA. Fifteen females ingesting a diet containing 0.06 percent 2-FAA for an average of 6.7 months were studied (table 2): Six developed carcinomas of the liver of which 5 had trabecular carcinomas, and one each of these had a cholangioma and an angiomatous hepatocellular carcinoma. The sixth animal had an angiomatous hepatocellular carcinoma. Histologically, these tumors were similar to those described in experiment 1. Mammary tumors were present in 6

animals, but 9 female rats fed the basal diet without carcinogen for an average of 10.4 months developed no liver or mammary tumors.

Acute Experiments

To observe the early morphologic changes in the livers of male and female rats, we killed groups of rats on the diet containing 0.03 percent 2-FAA at monthly intervals for 4 months. Since the livers of male rats but not female rats showed early morphologic changes after 1 month, groups of male rats in another experiment were killed at weekly intervals for the first 4 weeks.

The dietary and carcinogen intake and the body and liver weights of animals killed at weekly and monthly intervals are summarized in table 3. The average daily intake of diet and carcinogen for each sex is similar for the comparable time intervals. However, the males lost more weight than the females for the first 8 weeks, and by 12 and 16 weeks the females had gained while males weighed still less than they did initially. The liver weight corrected for body weight at the time of killing was somewhat less in the males than in the females killed at 4 and 8 weeks, but by 12 and 16 weeks the liver weight per unit body weight was much heavier in the males than in the females.

Morphologic Changes in Livers of Male Rats

One week.—The only histologic change observed in 4 rats was an occasional mitotic figure in bile-duct cells (fig. 11). The hepatic cells appeared normal.

Two weeks.—Along with occasional mitoses of bile-duct cells in the livers of 4 rats, a few additional changes were observed. Three of 4 animals showed the presence of a few "oval cells" (*6*) in the portal areas of the liver (fig. 12). These cells had pale blue-staining nuclei and lightly basophilic cytoplasm. The nuclei had a prominent nucleolus and a fine chromatin network. The nuclear membrane was clearly outlined while the cell boundary was indistinct. In addition, livers from 2 of the 3 animals revealed occasional focal areas of nodular hyperplasia. The liver of 1 of the 4 animals had no abnormal histologic changes.

Three weeks.—The livers of all 4 animals in this group had occasional mitotic figures of bile-duct cells with an increased number of oval cells in the portal areas of the liver. The liver of 1 animal had an area of nodular hyperplasia.

Four weeks.—The livers of the 9 animals in this group had occasional mitotic figures in bile-duct cells (fig. 13), an increased number of oval cells (figs. 14 and 15) with mitotic figures in these cells (fig. 16) in a few cases, and from few to many areas of nodular hyperplasia (figs. 17 and 18). In some livers the oval cells extended from the portal areas toward the central veins and involved from one half to three fourths of the lobule (fig. 14). In two livers the areas of nodular hyperplasia showed marked

Table 3.—Diet and 2-FAA intake and body and liver weights in male and female rats killed at weekly and monthly intervals

Duration (*weeks*)	Sex	Number of rats	Diet intake g/rat/day	2-FAA intake mg/rat/day	Body weight			Liver weight	
					Initial (g)	Terminal (g)	Change (g)	Gram	g/100 g terminal body weight
1	M	4	6.4 ± 0.4*	1.9 ± 0.1*	245 ± 5*	232 ± 1*	−13	9.5 ± 0.6*	4.1 ± 0.3*
2	M	4	7.3 ± 0.8	2.2 ± 0.2	249 ± 11	223 ± 9	−26	8.3 ± 0.8	3.7 ± 0.2
3	M	4	7.9 ± 0.7	2.4 ± 0.2	239 ± 9	211 ± 4	−28	8.6 ± 0.2	4.1 ± 0.1
4	M	9	6.5 ± 0.3	1.9 ± 0.1	223 ± 12	201 ± 12	−22	7.0 ± 0.6	3.4 ± 0.2
8	M	3	7.2 ± 0.4	2.2 ± 0.1	271 ± 7	248 ± 12	−23	9.3 ± 1.1	3.7 ± 0.2
12	M	3	7.4 ± 0.6	2.2 ± 0.2	226 ± 31	222 ± 28	−4	12.6 ± 2.1	5.6 ± 0.5
16	M	4	7.2 ± 0.5	2.2 ± 0.2	230 ± 20	212 ± 42	−18	13.3 ± 3.0	6.0 ± 0.7
4	F	3	6.3 ± 0.6	1.9 ± 0.2	191 ± 11	179 ± 10	−12	7.2 ± 0.7	4.0 ± 0.2
8	F	3	6.1 ± 1.5	1.8 ± 0.5	170 ± 5	158 ± 26	−12	6.7 ± 0.7	4.3 ± 0.4
12	F	3	7.0 ± 0.8	2.1 ± 0.2	172 ± 12	190 ± 9	+18	8.1 ± 1.1	4.2 ± 0.4
16	F	4	7.3 ± 0.5	2.2 ± 0.1	170 ± 7	201 ± 10	+31	9.8 ± 1.4	4.8 ± 0.5

*Mean ± standard error of the mean, calculated by the method of Mantel (5).

cellular pleomorphism with frequent mitotic figures. These were considered to demonstrate atypical changes. In a few livers infrequent mitotic figures in hepatic cells were observed (fig. 19).

Two months.—The sections of livers of 3 male rats showed a moderate increase of oval cells predominantly in the portal areas. Occasional mitotic figures were present among the oval cells. The number of oval cells appeared to be somewhat less than at 1 month, and these cells were compressed together into columns at the periphery of many areas of nodular hyperplasia (fig. 20). Some hepatic cells and also pigment-laden macrophages were present among the oval cells. The macrophages contained yellowish to brownish granules. On paraffin sections some of the granules stained with acid-fast, periodic acid-Schiff, and Sudan IV, while others stained with an iron stain. Similar pigment granules were observed in macrophages in the livers of the chronic experiments. The normal lobular pattern of the livers was distorted by the multiple areas of nodular hyperplasia (fig. 20). In one liver the areas of hyperplasia showed cellular atypism. Two livers had mild fatty change and one had a few cysts.

Three months.—Oval cells were present in the livers of 3 rats in similar arrangement but in fewer number than that found in the livers at 2 months. Many areas of nodular hyperplasia, larger than those found previously, were present throughout the livers. Some of these showed atypical changes. Although there was a marked distortion in the normal lobular pattern of the livers by the hyperplastic nodules, no significant increase of fibrous tissue was found with special stains, such as the Mallory phosphotungstic-acid hematoxylin, Masson trichrome, and van Gieson. Two livers had focal areas of cholangiofibrosis.

Four months.—The livers of 4 animals resembled those seen at 3 months and had oval cells, cholangiofibrosis, and many nodular hyperplastic areas which contained some atypical changes. Three livers had cystic areas and 2 had fatty change. One liver contained early trabecular carcinoma.

Morphologic Changes in Livers of Female Rats

One month.—The livers of 3 female rats had occasional mitotic figures in bile-duct cells. Although no exact quantitative study was made, it was apparent on inspection that there were fewer mitotic figures in bile-duct cells in the livers of females than in the livers of male rats after 1 month. Mild, diffuse, fatty change was noted in the livers of 2 animals. No other changes were observed.

Two months.—The livers of 2 animals contained mild, diffuse, fatty change. One animal showed many oval cells in the portal area of the liver and a few areas of nodular hyperplasia. This animal had lost 60 g in 2 months, while each of the other 2 animals in this group had gained 12 g during the same interval. The diet and carcinogen consumption of this animal was approximately half that of each of the other 2. Of all the experimental groups of female rats, this animal had the largest number

of oval cells in the liver and the changes resembled those found in the livers of male rats at 1 to 2 months.

Three months.—The livers of 3 rats had mild to moderate diffuse, fatty change. Livers of 2 rats had a few small, pale-staining areas of nodular hyperplasia (fig. 21). A few oval cells were present in the portal areas of the livers in 2 of the 3 rats. The number of oval cells in the livers of these animals could quantitatively be compared with the number of oval cells in the livers of male rats at 2 weeks.

Four months.—The livers of 4 rats had mild fatty change and also occasional areas of nodular hyperplasia. Oval cells similar in number to those found in female rats at 3 months were in 4 livers. No other changes were observed.

Control animals.—Livers from male and female rats fed the basal diet and killed at the different intervals were examined. Some of these animals were fed a basal diet containing low pyridoxine·HCl (0.25μg/g) diet. Sections of livers from these rats were essentially normal. No mitotic figures of bile-duct cells were observed in any of the livers.

The histopathologic changes in the livers of male and female rats ingesting a diet containing 2-FAA or a control diet and killed at early intervals up to 4 months are summarized in table 4.

Since the morphologic changes in the livers of female rats killed at intervals for 4 months were not as developed as those in the males for the same intervals, we decided to ascertain what further changes developed in the following months in female animals. For this reason the livers of 5 female rats of experiment 1 of table 2 that died or were killed at 4.6 to 5.8 months were re-examined and are described here. In all 5 livers there was a small number of oval cells in the portal areas. These changes were comparable to those in males at 3 weeks. Oval cells and prominent bile ductules were present in small localized areas but without extension as seen in the livers of males, as for example, at 4 weeks or later. All 5 livers had a moderate degree of fatty change. Three livers had areas of nodular hyperplasia, but no distortion of lobular pattern was observed in any of these livers. Pigment-laden macrophages were observed in 3 livers.

Influence of Exogenous Testosterone on Early Morphologic Changes in Liver

An experiment was designed to evaluate the influence of testosterone on the early histologic changes in the liver. The diet and carcinogen intake, body weight, and liver weight of male and female rats are summarized in table 5.

The animals were divided into 7 groups and were killed at 1, 2, and 4 months. The effects in the control animals, intact male and female rats ingesting the diet containing 0.03 percent 2-FAA (groups 1 and 3), were similar to those noted with animals described earlier for the same intervals (table 3). The main difference was that the animals of these 2

TABLE 4.—Histopathologic changes of livers of male and female rats fed a diet containing 0.03 percent 2-FAA and killed after intervals of 1 to 16 weeks

Duration of experiment (*weeks*)	Sex	Number of rats	Number of rats showing histologic changes in liver							
			Mitoses in bile-duct cells*	Oval cells	Fatty change	Nodular hyperplasia	Cellular atypism	Cholangio-fibrosis	Cysts	Carcinoma
1	M	4	4							
2	M	4	4	3		2				
3	M	4	4	4		1				
4	M	9	9	9	2	9	2			
8	M	3		3	2	3	1		1	
12	M	3		3	3	3	3	2	3	
16	M	4		4	2	4	4	4	3	1
4	F	3	3		2					
8	F	3†		1	2	1				
12	F	3		2	3	2				
16	F	4		4	4	4				
Controls	M	4	0							
	F	6	0		2					

*Examination for bile-duct mitosis was carried out only in livers of animals on the diet containing 2-FAA up to and including 4 weeks and in all livers of control animals.

†*See* text for description on one female rat in this group.

TABLE 5.—Influence of exogenous testosterone on rats ingesting 2-FAA

Group	Duration (*months*)	Sex	Number of rats	Conditions	Body weight (g)			Diet intake g/rat/day	2-FAA intake mg/rat/day	Liver weight	
					Initial	Terminal	Change			Grams	g/100 g terminal body weight
1	1	F	3	—	142 ± 4*	147 ± 4*	+5	6.3 ± 0.4*	1.9 ± 0.1*	6.3 ± 0.4*	4.3 ± 0.3*
	2	F	3	—	135 ± 1	148 ± 10	+13	6.5 ± 0.5	1.9 ± 0.1	7.0 ± 1.0	4.7 ± 0.4
	4	F	3	—	136 ± 10	178 ± 8	+42	7.3 ± 0.5	2.2 ± 0.2	9.2 ± 0.2	5.2 ± 0.2
2	1	F	3	Testosterone†	135 ± 4	151 ± 9	+16	5.4 ± 0.5	1.6 ± 0.2	6.6 ± 0.3	4.3 ± 0.1
	2	F	3	Testosterone	141 ± 5	157 ± 17	+15	6.7 ± 1.0	2.0 ± 0.3	7.3 ± 1.0	4.6 ± 0.1
	4	F	4	Testosterone	144 ± 3	158 ± 10	+14	7.4 ± 0.5	2.2 ± 0.1	10.7 ± 0.7	6.8 ± 0.4
3	1	M	3	—	182 ± 9	212 ± 14	+30	8.6 ± 0.9	2.6 ± 0.3	8.6 ± 1.0	4.1 ± 0.2
	2	M	3	—	159 ± 6	204 ± 9	+45	8.5 ± 0.4	2.5 ± 0.1	9.6 ± 0.3	4.7 ± 0.2
	4	M	3	—	175 ± 4	200 ± 8	+25	7.4 ± 0.5	2.2 ± 0.1	11.7 ± 0.3	5.9 ± 0.2
4	1	M	5	Testosterone†	173 ± 2	175 ± 9	+2	7.0 ± 0.4	2.1 ± 0.1	6.4 ± 0.4	3.7 ± 0.1
	2	M	3	Testosterone	174 ± 17	188 ± 15	+14	6.7 ± 0.6	2.0 ± 0.2	7.7 ± 0.7	4.1 ± 0.1
	4	M	2	Testosterone	185 ± 5	152 ± 8	−33	7.7 ± 1.2	2.3 ± 0.4	10.2 ± 0.5	6.7 ± 0.1
5	1	M	4	Castrated	171 ± 10	192 ± 19	+21	7.6 ± 0.4	2.3 ± 0.1	8.3 ± 1.5	4.2 ± 0.4
	2	M	3	Castrated	164 ± 3	229 ± 13	+65	8.1 ± 0.5	2.4 ± 0.2	9.9 ± 1.1	4.3 ± 0.2
	4	M	3	Castrated	189 ± 10	211 ± 21	+22	8.2 ± 0.5	2.5 ± 0.1	10.4 ± 1.3	4.9 ± 0.4
6	1	M	3	Castrated + testosterone†	182 ± 5	179 ± 16	−3	6.1 ± 0.5	1.8 ± 0.1	7.4 ± 0.7	4.1 ± 0.1
	2	M	3	"	173 ± 15	191 ± 15	+18	6.8 ± 0.5	2.0 ± 0.2	8.6 ± 0.8	4.8 ± 0.1
	4	M	4	"	183 ± 9	170 ± 5	−13	6.5 ± 0.2	2.0 ± 0.1	10.7 ± 0.5	6.3 ± 0.3
7	1	M	3	Castrated + oral testosterone‡	170 ± 10	185 ± 14	+15	7.3 ± 0.3	2.2 ± 0.1	6.6 ± 0.9	3.7 ± 0
	2	M	4	"	168 ± 9	147 ± 11	−21	6.2 ± 0.3	1.9 ± 0.1	6.0 ± 1.2	4.0 ± 0.6
	4	M	2	"	177 ± 21	133 ± 29	−44	6.9 ± 0.2	2.1 ± 0	8.4 ± 3.0	6.2 ± 0.5

*Mean ± standard error of the mean, calculated by the method of Mantel (*8*).

†Rats in group 2, 4, and 6 received 500 μg testosterone proprionate in sesame oil subcutaneously daily for 5 days each week.

‡Rats in group 7 received methyl testosterone in the diet (1.67 mg/kg).

groups gained weight, while in the earlier experiment they lost weight during comparable time intervals. This result may be related to the younger starting age of 2 months compared with the age of 2.5 to 3.1 months in the previous experiment. Female rats receiving 2-FAA in the diet and testosterone (group 2) by 4 months showed a marked increase in liver weight. These changes were similar to those in the intact males ingesting the 2-FAA diet (group 3). Male rats receiving 2-FAA in the diet and testosterone (group 4) by 4 months showed a marked increase in liver weight. The castrated males receiving 2-FAA in the diet (group 5) showed changes similar to the females on the same diet (group 1), while the castrated males receiving 2-FAA in the diet and testosterone either subcutaneously or in the diet (groups 6 and 7) showed liver changes similar to those in the intact males (group 3).

Histologic examination revealed that the livers of intact female and male rats in groups 1 and 3 were similar at 1, 2, and 4 months to those described earlier for intact female and male rats. The livers of females receiving testosterone (group 2) had areas of nodular hyperplasia at 2 and 4 months and oval cells at 4 months. The changes in the livers of male rats receiving testosterone (group 4) were more pronounced than the changes found in the livers of intact males (group 3). After 1 month atypical areas of nodular hyperplasia were present in the livers of rats of group 4, and 1 liver had early trabecular carcinoma. The livers of castrated males (group 5) were similar to those in intact females (group 1). The histology of the livers of castrated males receiving testosterone (groups 6 and 7) was similar to that in intact males (group 3).

DISCUSSION

Although many investigators (*4, 7–11*) have described the pathology of liver tumors in rats ingesting 2-FAA, no sex difference in the morphology of the tumors has previously been reported. In our present study we observed differences in the histology of the liver tumors that developed in male and female rats of the Buffalo strain. Morphologically the liver tumors in male rats were less differentiated and more anaplastic hepatocellular carcinomas than the liver tumors in the females, which could be interpreted as indicating a higher degree of malignancy of the liver tumors in male than in female rats. This view was supported biologically by the high incidence of lung metastases from liver carcinomas in the males.

Several investigators (*6, 7, 12*) have described the early histologic findings in the livers of rats ingesting diets containing 2-FAA. However, because we observed a sex difference in the histology of liver tumors in rats ingesting 2-FAA in our chronic experiments, we also investigated the sequence of early histologic changes in the livers of Buffalo-strain rats on the same diet.

The first histologic change found in the livers of male rats fed 2-FAA was the appearance of mitotic figures in bile-duct cells within 1 week. From 2 to 4 weeks, the periportal areas of the livers had oval cells which gradually increased in number and extended toward the central areas. By 3 to 4 weeks, areas of nodular hyperplasia appeared and enlarged progressively to distort the lobular pattern and increase the weight of the liver. By 3 months many of these areas of hyperplasia were histologically atypical. In contrast, while the livers of female rats contained mitotic figures in bile-duct cells at 1 month, only a few oval cells were present in the livers during the following 3 months and there was no marked increase up to 5.8 months. The most advanced changes in oval cells were similar to those found in males after 3 weeks. Small areas of nodular hyperplasia appeared by 2 to 3 months, but were never as numerous or as large as those in the livers of male rats.

Our early histologic findings in the livers of male and female rats ingesting a diet containing 2-FAA are in agreement with those previously reported by Farber (*6*) in studies up to 49 days. Whether oval cells or subsequent nodular hyperplasia might influence, directly or indirectly, tumorigenesis is not known, yet because of their close relationship, it is feasible to assume that oval cells may influence the process of hyperplasia.

Oval cells or cells considered to be similar to them (*4, 6, 13–24*) have been described in the liver after ingestion of carcinogenic agents, such as 3′-methyl-4-dimethylaminoazobenzene, 2-FAA, thioacetamide, dimethylnitrosamine, and ethionine, and in a variety of non-neoplastic conditions, such as those after bile-duct obstruction and in acute and subacute yellow atrophy, and after α-naphthyl-isothiocyanate ingestion. Disagreement exists regarding the origin of the cells (*4, 6, 13, 18, 19, 24, 25*), which can be resolved only by further study of their development under different conditions. Studies of these cells in livers of rats ingesting ethionine in the diet have demonstrated mitotic figures in bile-duct cells during their appearance and have suggested that these cells are derived from ductular epithelial cells (*6, 13, 18*). Our findings of bile-duct mitotic figures followed by the appearance of oval cells in the periportal areas of the livers of rats ingesting a diet containing 2-FAA are evidence in support of a bile-duct cell origin.

The early histologic changes in the livers of both sexes were influenced by the administration of testosterone. Although castrated males had liver changes similar to those seen in females, these males receiving testosterone had the same changes as were found in the intact males. Intact females receiving testosterone developed earlier and more extensive changes regularly found only in the livers of males. The observations reported here indicate that testosterone influences early histologic changes in the livers of male rats ingesting 2-FAA, and may be an accelerating factor in liver tumorigenesis induced by this carcinogen. Support for this view comes from earlier studies with rats of certain other strains ingesting 2-FAA (*26*) or diacetylaminofluorene (*27*) and receiving testosterone. In the former study testosterone accelerated and intensified the

hepatic lesions in male rats. In the latter study it was thought to be responsible for the higher incidence of liver tumors in male rats in comparison with female rats.

Our results with rats of the Buffalo strain fed 2-FAA in the diet indicate that under ordinary experimental conditions male rats develop a higher incidence of liver tumors than do female rats. Many other studies (*8, 26, 28–35*) have reported that the incidence of liver tumors in rats of many strains ingesting 2-FAA in the diet is higher in males than in females. The autopsy findings in our experiments raised the question whether the lower incidence of liver cancer in females in comparison with males was caused by the earlier deaths related to mammary cancers. In one experiment the length of life of female rats ingesting 2-FAA was prolonged by simple mastectomies as soon as mammary tumors developed. In this experiment the incidence of liver tumors in female rats was much higher than the incidence in our other experiments. In another study (*1*) in which female rats were fed a diet containing 2-FAA but low in pyridoxine, the animals survived longer, developed fewer mammary tumors and more liver tumors than other animals ingesting the same diet except high in pyridoxine. These findings suggest that, although liver tumors develop more slowly in females than in males, it is necessary to be cautious in interpreting a sex difference in over-all liver-tumor incidence.

It has been reported (*36*) that the administration of 2-FAA in high doses is more toxic to male than to female rats. This raised the question whether female rats ingesting a diet containing higher than 0.03 percent 2-FAA, as used in most of our experiments, would develop a higher incidence of liver tumors. In one chronic experiment in which female rats ingested a diet containing 0.06 percent 2-FAA, the incidence of liver tumors was essentially the same and not greater than in females ingesting a diet containing 0.03 percent 2-FAA. Likewise, the histologic appearance of the tumors was essentially the same. These findings indicate that the sex differences are not related to an effect of dosage within the range used.

Since it is reported that human males have a higher incidence of liver cancers than do females (*37*), it seems noteworthy that a similar sex difference apparently exists in rats ingesting the carcinogen 2-FAA. Since many other chemical carcinogens show no sex difference in rat liver tumorigenesis, 2-FAA is of interest in the study of liver-tumor induction in both male and female rats. This morphologic study indicates that 2-FAA offers a satisfactory procedure for the study of liver carcinogenesis. Further studies may ultimately help to elucidate the cause for a sex difference in the response of the livers of males and females to this carcinogen. Such basic information is vital for our ultimate understanding of liver carcinogenesis in experimental animals and probably also in man.

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PLATES

PLATE 8

FIGURE 1.—Liver of male rat fed diet containing 0.03 percent 2-FAA for 7.5 months. Anaplastic carcinoma with blood vessel containing tumor in *left center* area. Hematoxylin and eosin. × 70

FIGURE 2.—Higher magnification of preceding. *Note* cellular pleomorphism and some multinucleated tumor cells. Hematoxylin and eosin. × 340

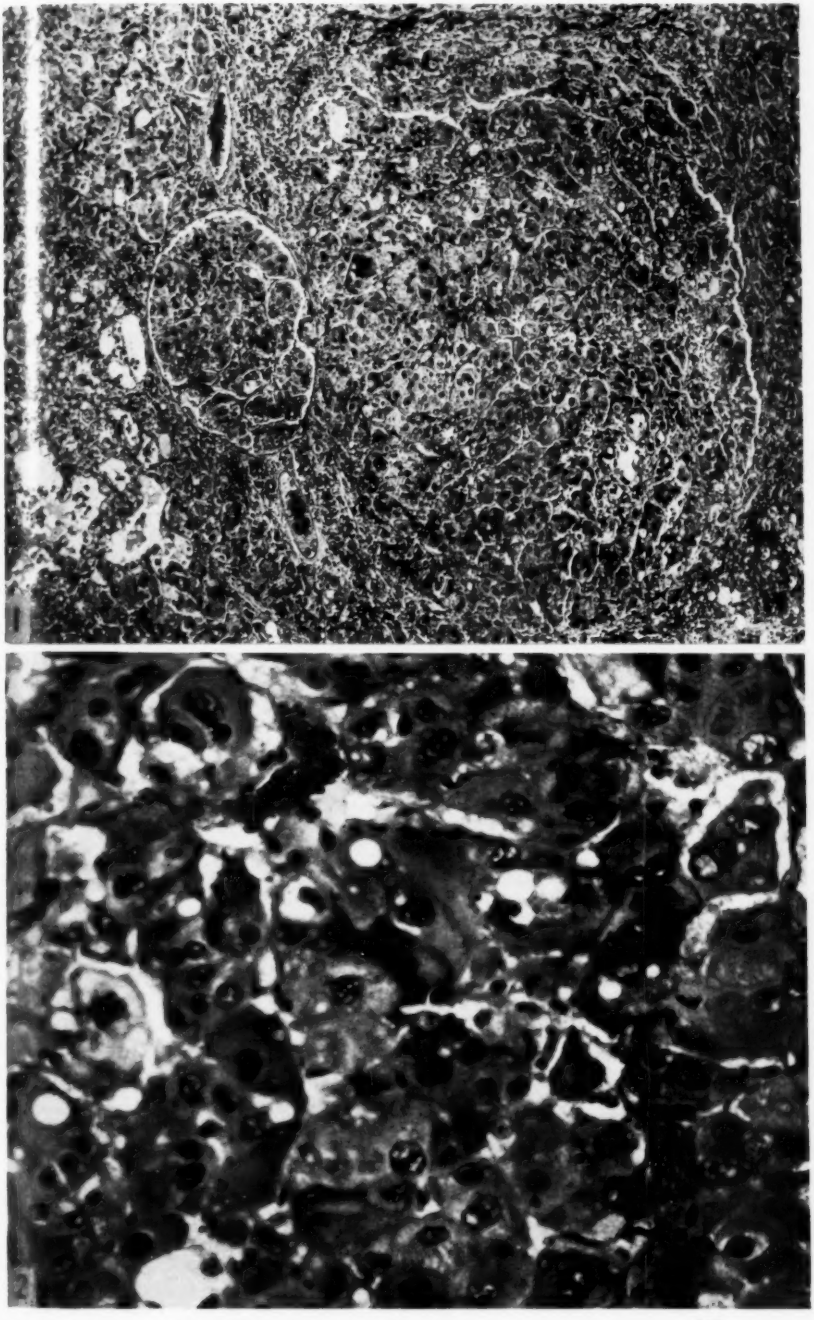
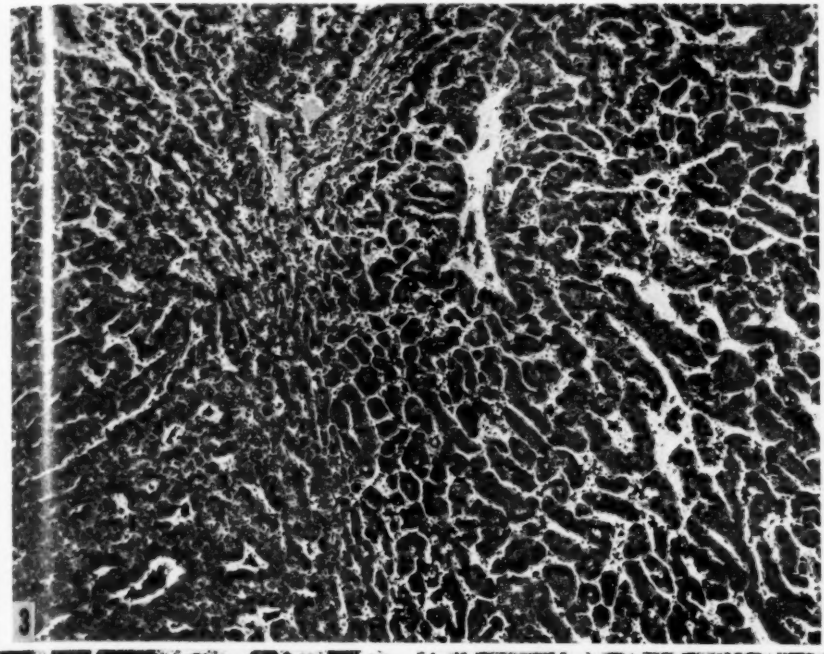


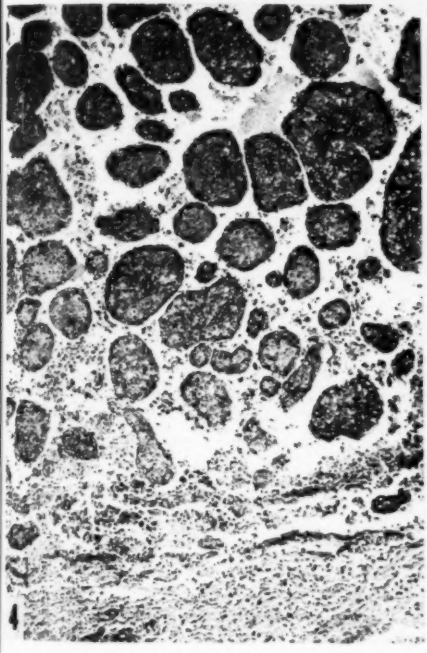
PLATE 9

FIGURE 3.—Liver of female rat fed diet containing 0.03 percent 2-FAA for 7.6 months. Trabecular carcinoma with double cords of liver cells and dilated sinusoids. Hematoxylin and eosin. × 70

FIGURE 4.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 7.7 months. Trabecular carcinoma with small and large cords of liver cells surrounded by vascular spaces. Hematoxylin and eosin. × 70

FIGURE 5.—Same rat as in figure 4. Lung metastasis resembling primary tumor. Hematoxylin and eosin. × 70





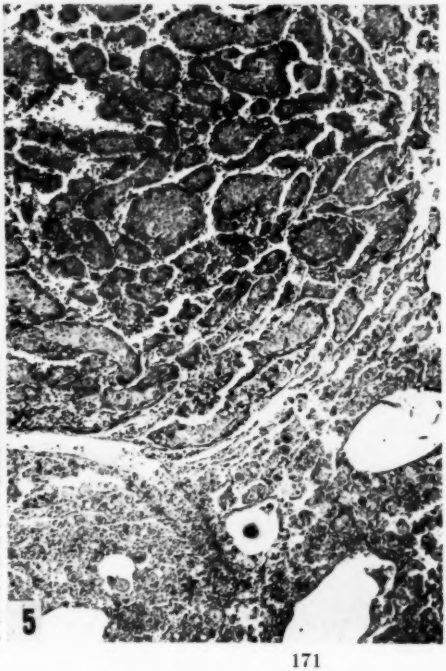


PLATE 10

FIGURE 6.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 7.5 months. Multiple cysts lined by cuboidal epithelium. Hematoxylin and eosin. × 40

FIGURE 7.—Liver from male rat fed diet containing 0.03 percent 2-FAA. Anaplastic carcinoma with adjacent hemorrhagic area *at left*. Hematoxylin and eosin. × 70

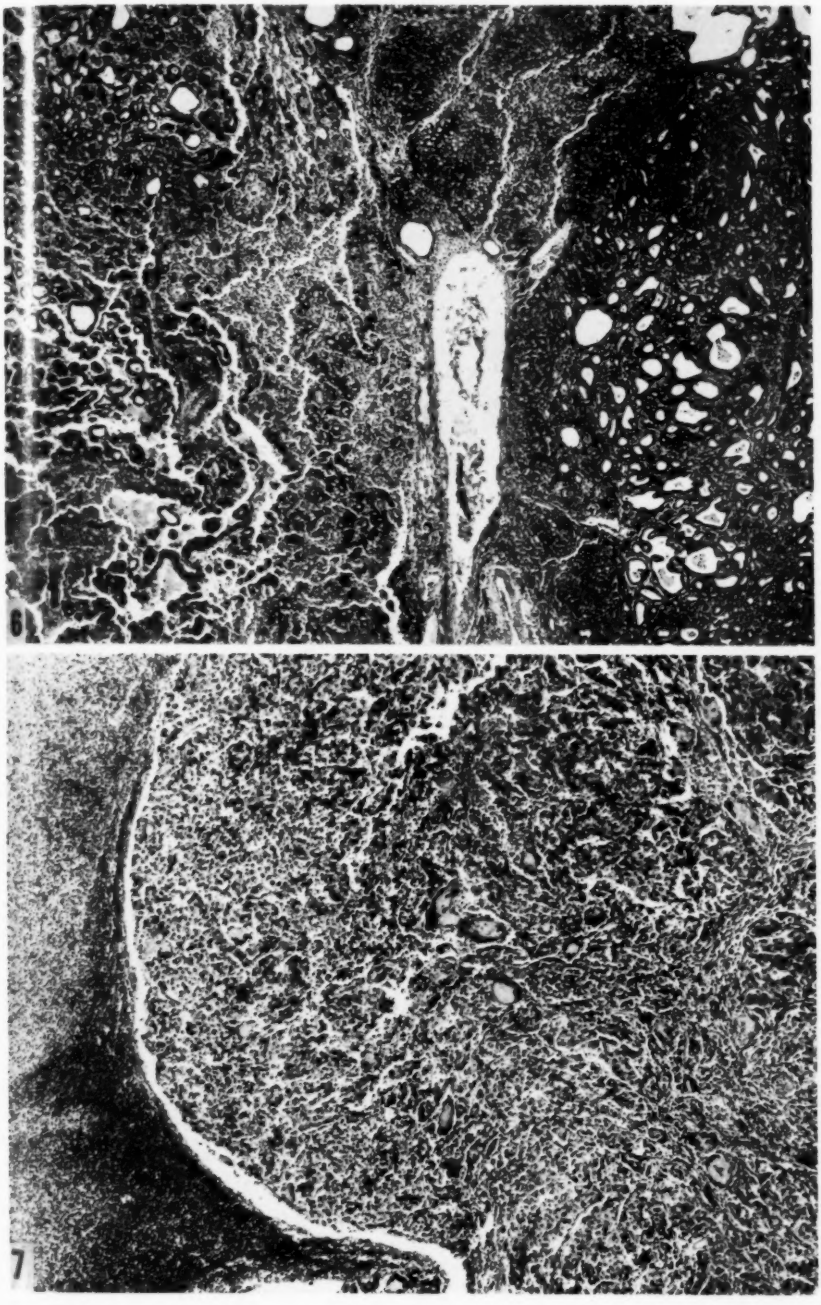


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PLATE 11

FIGURE 8.—Liver from female rat fed diet containing 0.03 percent 2-FAA for 7.8 months. Circumscribed tumor containing hemorrhage and necrosis in center and surrounded by tumor cells at periphery. Hematoxylin and eosin. × 55

FIGURE 9.—Liver from female rat fed diet containing 0.03 percent 2-FAA for 7.7 months. *Note* vascular spaces adjacent to tumor cells at periphery of hemorrhage. Hematoxylin and eosin. × 60

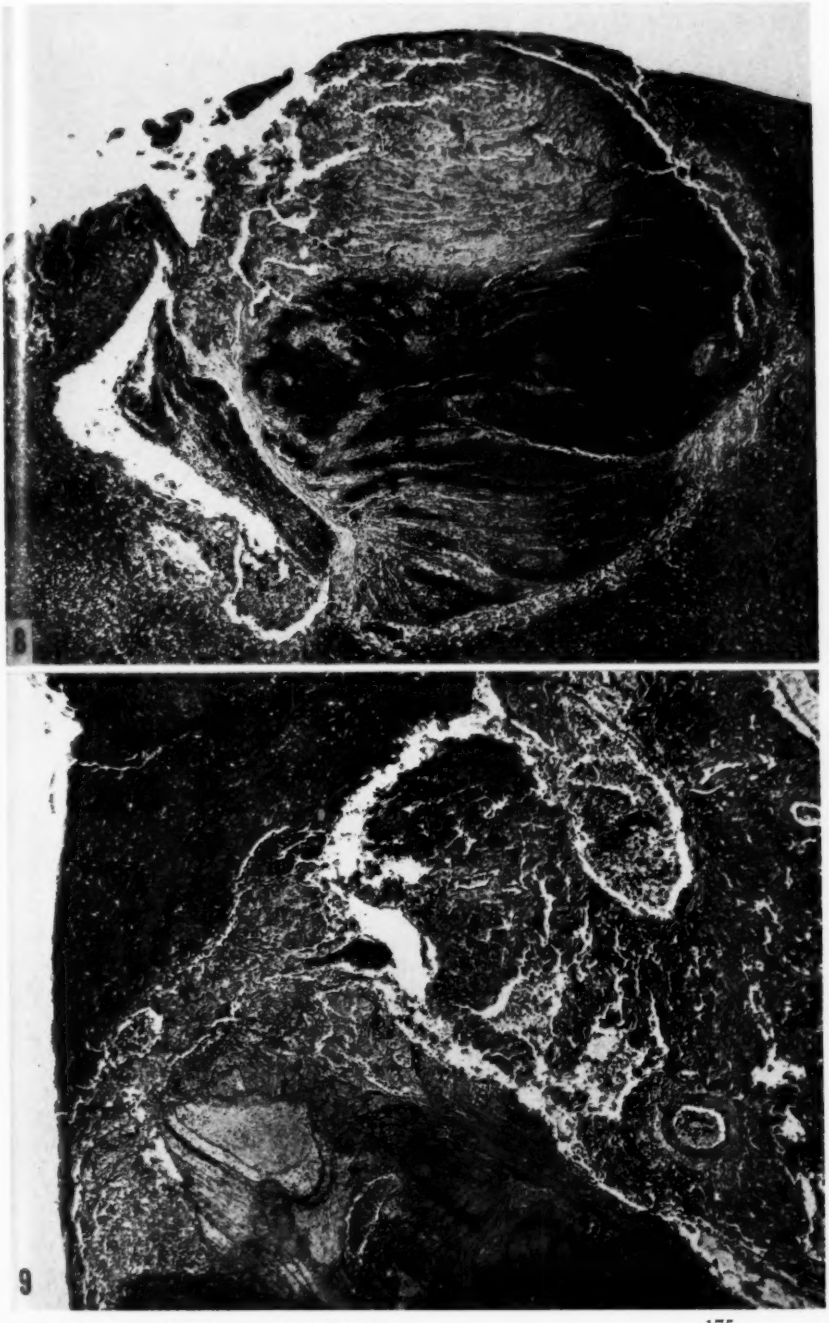


PLATE 12

FIGURE 10.—Higher magnification of preceding. *Note* pleomorphic tumor cells. Hematoxylin and eosin. × 210

FIGURE 11.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 1 week. A mitotic figure in bile duct. Hematoxylin and eosin. × 1,340

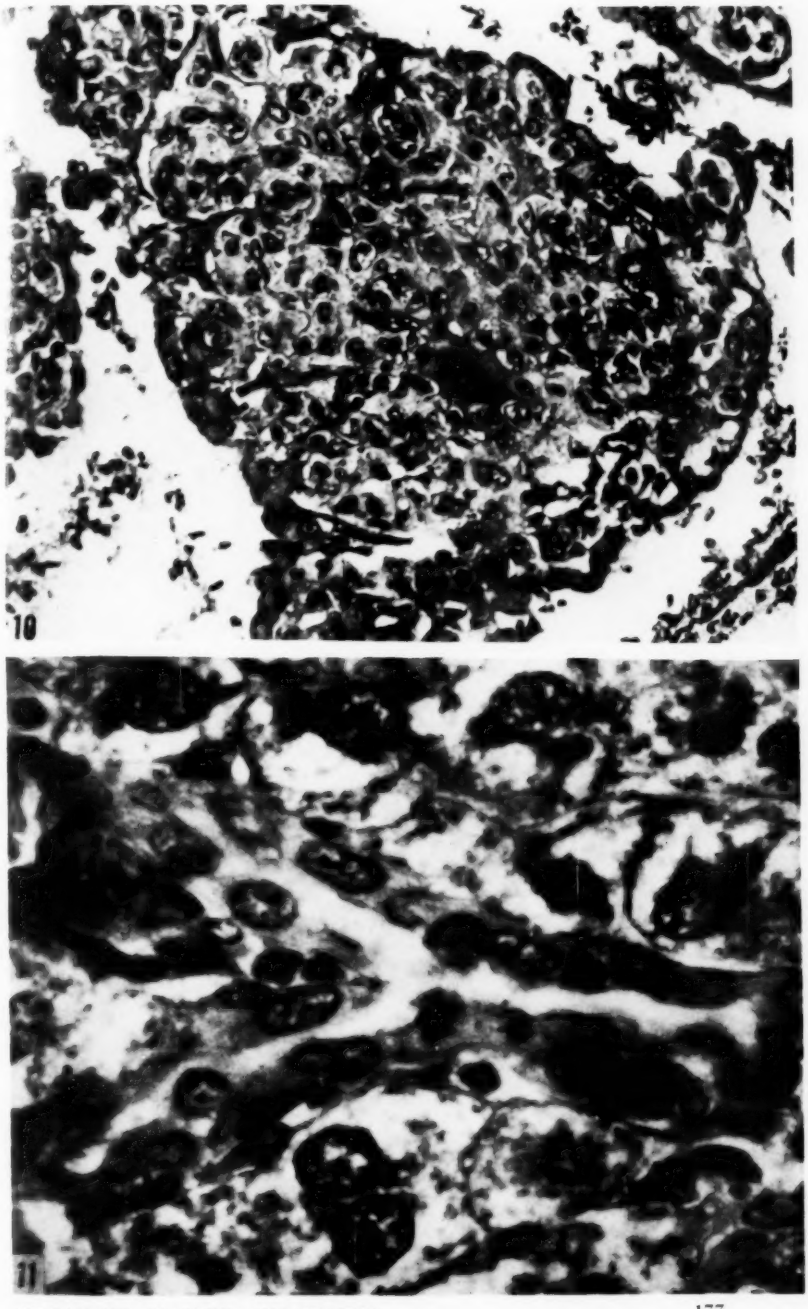
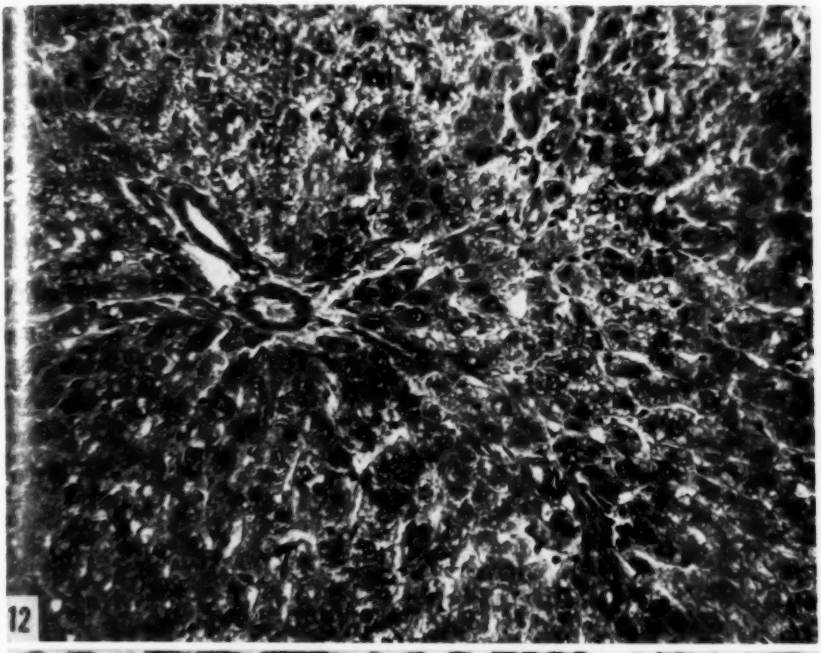


PLATE 13

FIGURE 12.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 2 weeks. Accumulation of oval cells in portal area. Hematoxylin and eosin. × 235

FIGURE 13.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 3 weeks. Mitotic figure within bile duct. Hematoxylin and eosin. × 1,340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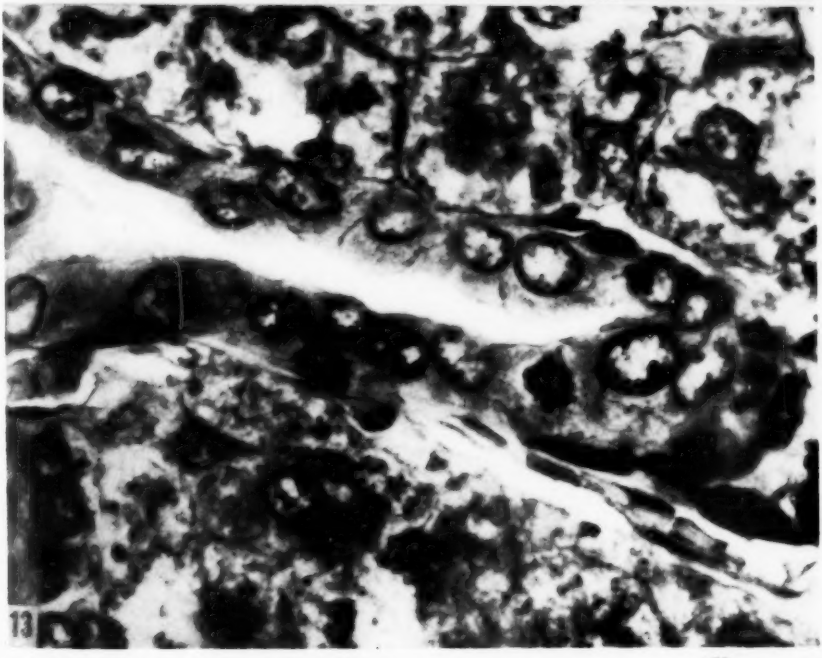
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PLATE 14

FIGURE 14.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 4 weeks. Numerous oval cells predominately in periportal areas of liver. Mallory's phosphotungstic acid-hematoxylin. × 60

FIGURE 15.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 4 weeks. Oval cells at higher magnification. Hematoxylin and eosin. × 200

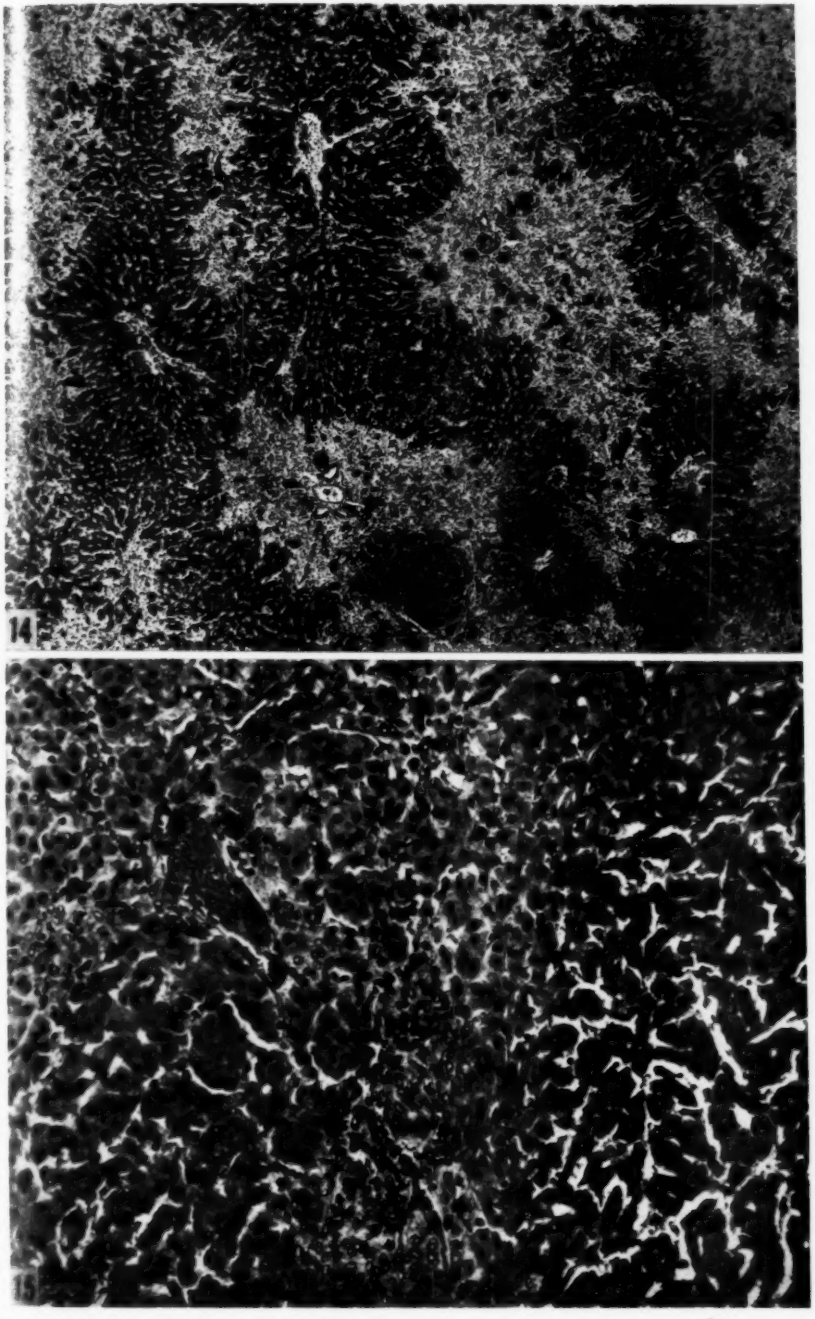

PLATE 15

FIGURE 16.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 4 weeks. Mitotic figures in oval cells. Hematoxylin and eosin. × 1,340

FIGURE 17.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 4 weeks Area of nodular hyperplasia surrounded by oval cells. Mallory's phosphotungstic acid-hematoxylin. × 150

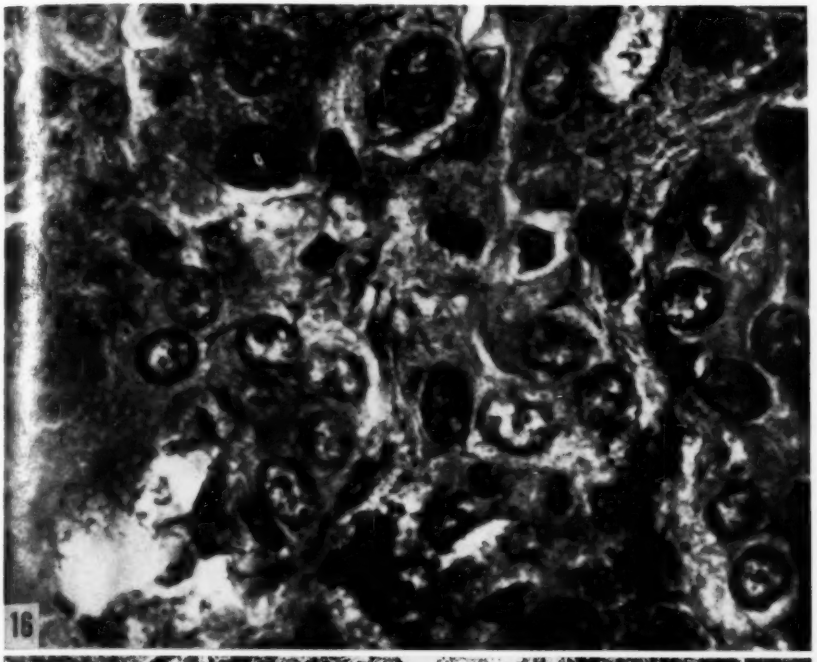
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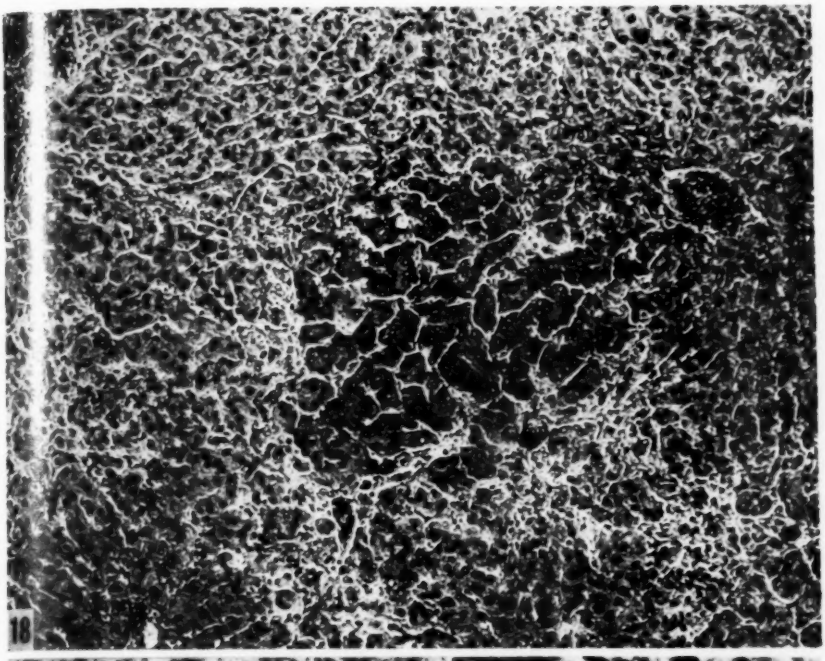
PLATE 16

FIGURE 18.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 4 weeks. Small area of hyperplasia. Hematoxylin and eosin. × 690

FIGURE 19.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 4 weeks. Several mitotic figures in hepatic cells. Hematoxylin and eosin. × 1,100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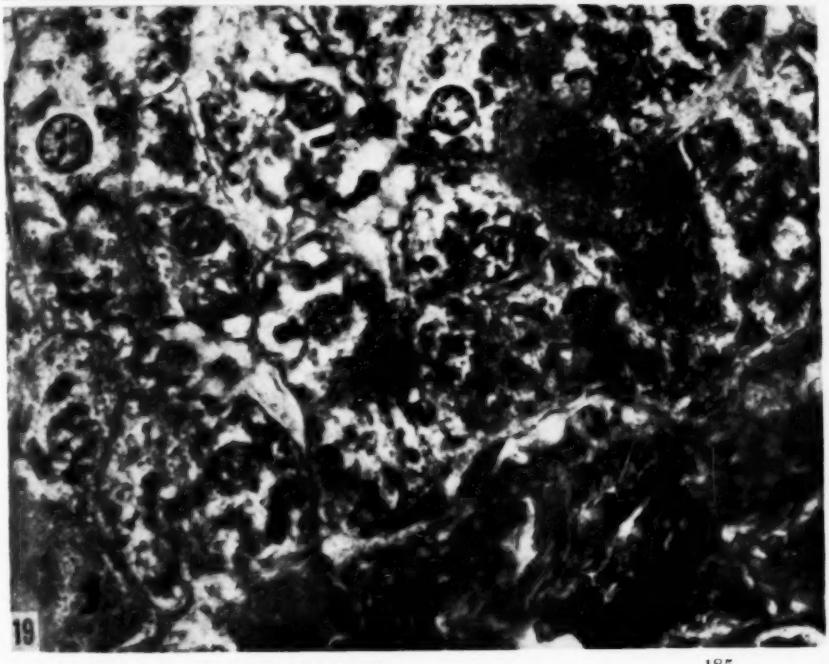
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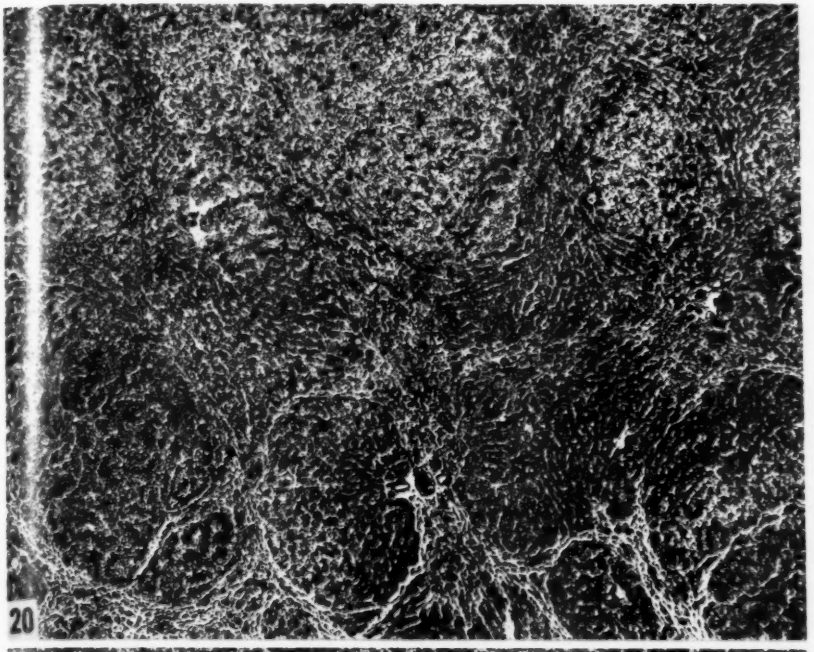
PLATE 17

FIGURE 20.—Liver from male rat fed diet containing 0.03 percent 2-FAA for 2 months. Multiple areas of nodular hyperplasia with distortion of normal lobular arrangement. Hematoxylin and eosin. × 60

FIGURE 21.—Liver from female rat fed diet containing 0.03 percent 2-FAA for 3 months. Small, pale area of hyperplasia. Hematoxylin and eosin. × 70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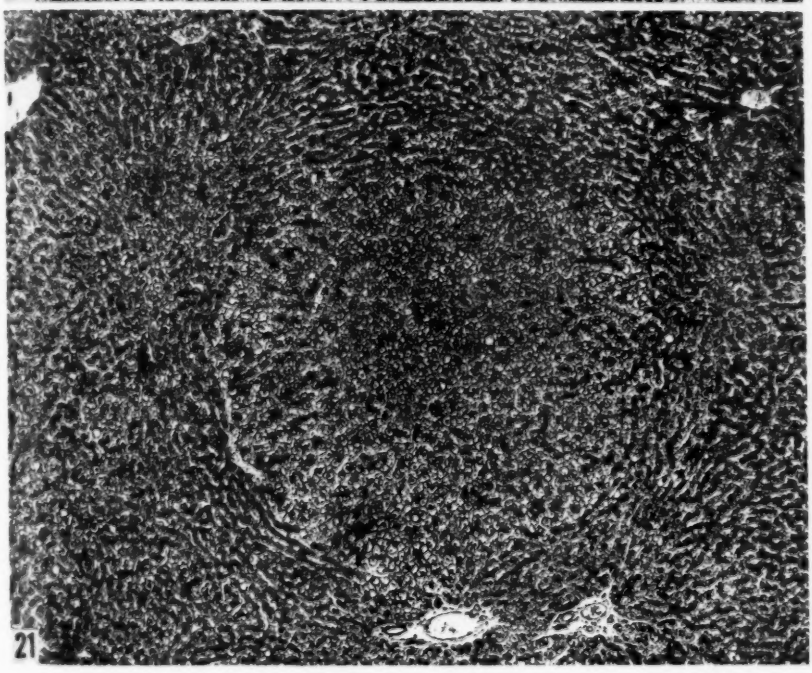
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Pathogenesis of a Virus-Induced Leukemia in Mice [1]

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SUMMARY

Male BALB/c mice that received a cell-free extract from the spleens of mice with leukemia induced by a virus were compared with BALB/c mice of the same age and sex that received a cell-free extract from normal spleens. Untreated BALB/c mice were used as controls. Mice 6 weeks of age were chosen for the injections, since tissues from newborn mice are hard to handle, and a relatively low dose of virus was given to insure a gradual development of leukemia. No difference in the three groups could be detected until 8 weeks had elapsed, when many megakaryocytes containing granulocytes were observed in the bone marrow of the virus-injected mice. The next difference detected was a gradually increasing hyperplasia of the spleen in the virus-injected mice. A few mice showed a localized reticulum-cell hyperplasia in the lymph nodes. The most significant alterations appeared to be in the thymus, and, with one exception, it was in this organ that leukemia was first recognized in the treated mice. The thymus in one leukemic mouse did not contain malignant cells but showed a granulomatous reaction. A series of transplants made from the primary leukemias resulted in lymphocytic neoplasms with different biologic characteristics in regard to growth period, spread from the site of inoculation, and organ involvement. The transfer of tissue from hyperplastic spleens from mice with no morphologic evidence of leukemia reproduced the same sequence of preleukemic lesions that were observed after transfer of the cell-free extract. This study indicates that inoculation of the leukemia-inducing virus causes a derangement in certain reticular organs of mice which is conducive to the development of lymphocytic neoplasms. The virus described by Moloney may be acting like many other carcinogenic agents. Several incidental lesions appearing in experimental, control, and stock BALB/c mice are described.—J. Nat. Cancer Inst. 26: 189–221, 1961.

FEW REPORTS are available describing the histologic sequences in leukemogenesis in laboratory mice. Potter, Victor, and Ward (*1*) described the histologic changes preceding spontaneous lymphatic leukemia in strain C58, and attached importance to a lesion in the lymph nodes. Kaplan (*2*) described the condition of the thymus in strain

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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C57BL mice killed near the end of the latent period in radiation-induced lymphoid tumors. McEndy, Boon, and Furth (*3*) reported that RF mice painted with methylcholanthrene showed hyperplastic changes in the lymph nodes and spleen, with large lymphoid follicles and many mitotic figures in the germinal centers. Arnesen (*4*) studied the preleukemic and early leukemic changes in the thymus gland of a high leukemia AKR/O strain, and described a preleukemic involution with a characteristic epithelial hyperplasia or neoplasia that was in sharp contrast to the age involution of the nonleukemic WLO strain thymus. In most other studies of leukemia in mice, only the final stage has been described.

The custom has been established of referring to neoplasms of the reticuloendothelial system and blood-forming organs in mice as "leukemia," regardless of whether neoplastic cells were proved to reach the peripheral blood (*5*). Leukemia has generally been used as a convenient, inclusive term, though such terms as lymphocytic neoplasm, lymphosarcoma, chloroma, and reticulum-cell sarcoma would often be more precise.

Mice receiving an injection of a cell-free extract of spleen from strain BALB/c mice with leukemia induced by a virus constituted the material for the present pathogenetic study. Because we wished to study any possible early effects of the virus, adult mice were chosen for the inoculation, since they are more satisfactory for histologic study than newborns. Also, because we hoped that the changes would develop gradually and not in an explosive fashion, the mice were given a relatively low dose of virus. Both the age of the inoculated mice and the size of the dose influence the time of development of leukemia. Under the conditions chosen for this experiment, all the virus-treated mice were expected to develop lymphocytic neoplasms within a limited period (*6*, *7*). Since the neoplasms could be induced in an inbred strain of mice, a control group of mice of the same strain, age, and sex, differing from the experimental mice only in the absence of the active virus from neoplastic tissue, was used for comparison. Because the experimental and control groups were so evenly matched, they could be directly compared, and when the labels on microscopic slides were hidden, organs from the experimental mice could be separated from the others whenever a significant histologic difference existed. Since an inbred strain was used, there was the further advantage that neoplasms from the treated mice could be transplanted, and the behavior and morphology of the resulting neoplastic lines could be observed.

It was hoped that a comparative study of the two groups of mice and the transplants would answer these questions:

1. Was there an early reaction after the injection of the leukemic spleen extract that might subside, yet leave a permanent alteration that could be detected histologically?
2. In what organs might differences in the control and experimental groups be found, and at what time would they develop?
3. At what site would the first positive neoplastic change appear?
4. What would be the nature of the resulting neoplasms? Would they

be identical, or would they differ in morphology and biological behavior?

5. Would there be a viral infection of normal cells and a direct malignant transformation, or would the infection produce some derangement, possibly hormonal, that would eventually lead to neoplasia?

MATERIALS AND METHODS

Virus-induced leukemic spleen tissue served as source material for the preparation of cell-free concentrates of the leukemia agent. The operative procedures and details of the method of differential centrifugation that were employed have been reported previously (*6*). The final concentration of the particulate fraction containing the active virus was 0.5 g equivalents per ml. A 1-g equivalent per ml concentrate is equal to 1.0 ml of final suspension for every gram of tissue processed.

Normal spleens, which served as control tissues, were harvested from mice of the same strain and approximately the same age as those animals that served as donors for the leukemic spleens. The normal tissues were extracted and the particulate fraction concentrated in a manner identical to that described for the leukemic tissues.

Test animals.—A total of 245 BALB/c mice 6 weeks of age was used. These animals were separated into 3 groups: experimental, control, and stock. Each of the 95 animals in the experimental group was inoculated intraperitoneally with 0.2 ml of a 10^{-1} dilution of the virus concentrate. The control group of 95 animals received, intraperitoneally, 0.2 ml of a 10^{-1} dilution of normal spleen extract. The remaining mice were not inoculated and were considered stock animals.

The mice of all groups were housed 5 to a cage, and the proposed date of autopsy was marked on each cage. The original plan was to autopsy the mice in matched experimental and control cages on days 1, 2, 3, 6, 9, 12, 15, and 21, and at weekly intervals thereafter. At the end of 3 weeks, however, when no differences in the 2 groups could be detected, it was decided to lengthen the intervals. The mice were therefore autopsied at weeks 5, 8, 11, 13, 14, 15, 16, 17, 20, and 21. After 13 weeks, usually 1 or 2 mice in each cage had died, and in the 14- and 16-week groups, 2 mice were added from cages scheduled for later dates, because they were not expected to survive until their scheduled date for autopsy. In addition to the control mice inoculated with normal spleen extract and the experimental mice inoculated with an extract from leukemic spleens, groups of 3 stock mice of the same strain, sex, and age were autopsied on days 1, 2, 6, and 16, and at weeks 5, 11, 15, 17, and 20. The 3 groups are subsequently referred to as control, experimental, and stock (table 1).

Autopsy procedure.—Each mouse was killed with ether immediately preceding autopsy. The same procedure was followed with the experimental, control, and stock mice. After the mouse was anesthetized, a blood smear was obtained from the tail. After death, the mouse was

TABLE 1.—Pathogenesis of leukemia in virus-treated mice

Weeks after inoculation	Number of mice	Number neoplastic	Limited neoplasm	Widespread neoplasm	Megakaryocytes with granulocytes	"Potter lesion" in lymph nodes	Hyperplasia of spleen	Thymus				
								Leukemic plus other organs	Reduced thymocytes	Leukemia in thymus only	Special	Section lost
8	—	5	—	—	3	—	—	—	4	—		1
11	2	3	2	—	4	—	—	—	2	2		1
13	2	3	1	1*	5	—	4	1	2	1		1
14	4	3	1	3	4	—	4	2	3	1	1†	—
15	—	3	—		3	1	3	—	2	—		1
16	4	3	1	3	2	—	4	3	4	1		—
17	1	3	1		4	—	4	—	2	1		—
20	3	1	1	2	3	1	3‡	2	2	1		—
21	2	2	1	1	2	1	4§	1	2	1		—

*When widespread neoplasm developed, magakaryocyte alteration and nonleukemic hyperplasia of spleen were usually obscured.

†Granulomatous lesion of thymus, widespread leukemia in other organs.

‡Average spleen weight 0.43 g.

§Average spleen weight 0.38 g.

pinned to a board, ventral side up, a midline incision through the skin made from chin to pubis, and the skin turned back and pinned to the board. Under a 6.6× lens of a dissecting microscope the peripheral lymph nodes and organs of the neck were examined. The abdominal cavity was opened, and a smear of the peritoneal fluid made. All abdominal and thoracic organs were examined; the skull was opened and the pituitary gland examined. The following organs and tissues were taken routinely for histologic examination: reticuloendothelial and blood forming: thymus, spleen, sternal bone marrow, and inguinal, cervical, and mesenteric lymph nodes; endocrine and reproductive: thyroid, adrenal and pituitary glands, and testis; other: salivary glands, brain, heart, lung, liver, kidney, and pancreas.

The tissues on removal were fixed in Zenker-formol and routinely stained with hematoxylin and eosin. The smears of blood and peritoneal fluid were colored with Wright's stain.

RESULTS OF POSTMORTEM EXAMINATION

Early Period

No difference in the control and experimental groups could be detected until 8 weeks had elapsed. Most of the lesions observed during the first 5 weeks were equally frequent in the experimental, control, and stock mice. These lesions, therefore, were considered to be unrelated to the experimental procedure. The following warrant comment:

Peripheral blood.—No direct erythrocyte, leukocyte, or platelet counts were made. The smears were inspected and an estimation made of the relative number of different types of leukocytes. The smears showed only minor variations in the experimental, control, and stock mice.

Peritoneal fluid.—In the experimental and control mice, during the first few days granulocytes and many histiocytes containing vacuoles and chromatin particles were frequently found. These were attributed to the reaction to the peritoneal injection. Evidence of phagocytic activity by histiocytes and granulocytes was rarely seen in the stock mice. At a later period, vacuolated histiocytes and granulocytes were seldom found in the experimental and control mice, and the smears were alike in all groups.

Heart.—The anterior surface in all three groups often showed an inflammatory reaction and calcification.

Parathyroid gland.—Small cysts lined by ciliated epithelium were often found.

Parathyroid gland region.—A frequent anomaly was the total or partial occupation of the site of the parathyroid gland by a lymphoid tissue identified as thymus (fig. 1).

Lymphoid tissue mass.—A minute structure made up of lymphoid cells was often seen in the cervical area. The cells composing the structure appeared larger and more deeply basophilic than were the lymphocytes

within a lymph node (fig. 2). The architecture also differed from a normal node, since there were no secondary centers, and a marginal sinus was tightly packed with deeply basophilic round cells (fig. 3). These lymphoid masses were at first presumed to be aberrant thymus tissue, since they were usually found in the cervical area, but one was later found near the renal lymph node.

Liver.—In the control, experimental, and stock mice, the liver showed frequent mitotic figures in the parenchymal cells during the first 2 weeks. Small areas of focal necrosis were also common, and occasionally there was a larger area resembling an infarct. In the later period, mitotic figures were seen less often, but areas of focal necrosis could still be found (fig. 4).

No morphologic difference could be detected between the control and experimental mice. A number of lesions observed during this early period and recurring throughout the periods of observation were considered to be unrelated to the treatment, since they were equally frequent in the experimental, control, and stock mice.

Later Period

Beginning at 8 weeks, and becoming progressively more definite in succeeding weeks, lesions were found in certain organs of the experimental mice. Those organs in which no differences could be detected, unless a widespread lymphocytic neoplasm had developed in the experimental mice, include brain, salivary glands—no lesions were ever found suggesting activity of the parotid-tumor agent—(*8*), heart, lungs, liver, pancreas, adrenal and thyroid glands, and testis. Although pituitary glands were fixed and imbedded, none were sectioned, because at the completion of this study it seemed improbable that the development of lymphocytic neoplasms was related to an endocrine disturbance.

The reticuloendothelial organs of the experimental group include bone marrow, lymph nodes, spleen, and thymus. These are described in what is considered to be an increasing order of importance, for it was in this system that positive differences were detected.

Bone marrow.—At 8 weeks, a difference in the megakaryocytes of the bone marrow was noted, and this difference persisted with varying frequency throughout the remaining period of observation. In the sternal marrow sections from 3 mice in the 8-week group, occasional megakaryocytes contained one or more granulocytes within the cytoplasm. Only a few of the megakaryocytes were thus altered, and more than one granulocyte inclusion was rare (figs. 5 through 9). This megakaryocyte alteration was not a positive criterion for the identification of the experimental mice, since it was found in only a small percentage of megakaryocytes, and there was occasionally a granulocyte inclusion in the control and stock mice. However, an accurate selection of bone marrow sections from the experimental mice was usually possible because of the change in the megakaryocytes.

Lymph nodes.—The lymph nodes of mice are extremely labile. A wide range is found in the activity in secondary centers, and varying degrees of reticulum-cell hyperplasia are common. Both the control and experimental groups in the present experiment showed many variations, and, with a few exceptions, no separation of the 2 groups could be made before leukemia developed. However, in 3 experimental mice, from the 15-, 20-, and 21-week group, respectively, one or more lymph nodes showed an area in which nests of hyperplastic reticulum cells appeared in the midst of normal lymphocytes (fig. 11). No other organs in these mice were leukemic. In mice in which leukemia had developed, leukemic involvement of the lymph nodes varied greatly, and even when a widespread leukemia developed, it was usual for some of the lymph nodes to be spared. Occasionally only a portion of a node was obscured by leukemic infiltration. The peripheral nodes were less often involved than the visceral lymph nodes.

Spleen.—The spleen was probably the most uniformly and consistently altered of all the organs examined. Enlargement of the spleen preceded the onset of leukemia and did not appear to be directly related to the neoplastic changes. We regret that the weight of the spleen was not recorded before the 20- and 21-week period, and we had to rely on a visual estimation of its size based upon a comparison of spleens from the experimental and the control groups. At 13 weeks, the spleens of the experimental mice were enlarged in comparison with the controls, and the size increased progressively until the 20-week period, when weights were taken and the average was 3 to 4 times the usual weight of the spleen in the mouse. When widespread leukemia developed, the size of the spleen increased enormously to 10 or more times the normal. Histologically, the hypertrophied preleukemic spleen showed generalized hyperplasia of all cell elements. This hyperplasia was evenly balanced and the normal architecture was maintained. Although there appeared to be an increase in megakaryocytes, this may have been deceptive, because the distinctive morphology of these cells strikes the eye. The megakaryocytes of the spleen occasionally contained granulocytes, but this feature was much less frequently detected in the spleen than in the bone marrow (fig. 10). The hyperplasia of the spleen in the experimental mice was sufficiently prominent and definite that a separation of experimental from control mice could be made after the 13-week period (figs. 12 and 13).

Thymus.—The thymus proved to be the site at which a recognizable neoplastic change nearly always began. Since the thymus was not put into a separate bottle for processing, and was embedded along with other small organs, no thymus tissue, or only a small area of thymus, was recovered in the completed microscopic material from a few autopsies. Enough thymus sections were available, however, to make it certain that the thymus is particularly significant in the genesis of leukemia in these mice. Serial sections were made from the blocks containing the thymus in 4 mice. These revealed a considerable variation at different levels in

the thymus gland, and it was evident that all parts of the organ did not simultaneously undergo a uniform alteration.

Among the 18 mice in which neoplastic changes had occurred, in only one was the thymus found to be free of neoplastic cells while other organs were involved. In 8 of the 18 mice, only the thymus showed neoplastic cells, while all other organs appeared to be free of leukemia. In the remaining 9 mice with such advanced leukemic changes that the site of origin was obscured, the thymus may have been the first site, for it was always greatly enlarged as a result of leukemic infiltration. Because the histologic alterations in the thymus were not uniform, it will be convenient to describe them as non-neoplastic, early neoplastic, and completely neoplastic.

The simplest non-neoplastic alteration was a moderate reduction in thymocytes that produced a relative prominence of the medulla, which in limited areas might replace the cortex and reach the capsule (fig. 14). A similar but less extensive change was seen occasionally in the control mice. This slight thymic atrophy was first detected at 8 weeks. Occasionally when the process was more advanced, no cortex was recognized. In 2 mice the degree of atrophy differed in the thymic lobes, one showing extreme atrophy, while the other still contained many thymocytes (fig. 15).

The most remarkable thymic alteration appeared in a mouse in the 14-week group, the only mouse in which lymphocytic neoplasia involved other organs but was absent in the thymus. In some areas the normal architecture of the thymus was obscured, and the thymic tissue was replaced by pale, fusiform cells, in which nests of large, foam cells with acicular space were found (figs. 16 and 17). This alteration was more prominent in one lobe, and serial sections showed variations in degree at different levels. Other organs in this mouse showed leukemia.

The earliest neoplastic change appeared in 2 mice at 11 weeks, where a limited area in the thymus showed leukemic cells interspersed with large macrophages containing chromatin particles. The bulk of the organ was only slightly increased, and neoplastic cells had not penetrated beyond the capsule (fig. 18). No other organs showed a neoplastic change at this time. A similar early change was found in 1 mouse at 13 weeks. In 3 of the 5 mice in which the leukemic change was limited to the thymus, involvement of 1 lobe only was noted, and the other lobe was atrophied (fig. 19).

The neoplastic change beginning in the thymus appeared to spread rapidly to the mediastinal tissues (fig. 20) and also involved the spleen, lymph nodes, bone marrow, and other organs. In some cases the previous outline of the thymus could be detected, and the neoplastic tissue within the thymus often contained more macrophages with vacuoles and chromatin particles than the tissue immediately beneath the capsule (fig. 21).

One thymus with leukemic cells showed small, irregular, eosinophilic masses within the nucleus of large, pale cells, probably thymus epithelial cells. No nucleoli were found in these cells. These intranuclear eosino-

philic masses were the only morphologic structures found in any of the tissues to suggest a virus effect (fig. 22).

Histology of the resulting neoplasms.—All the neoplasms developing in the virus-treated mice appeared to be of the lymphocytic type (figs. 23, 24, and 25). The cells were larger than the normal lymphocyte; the nuclei were hyperchromatic, round, or slightly indented, and there was a narrow rim of basophilic cytoplasm. Large phagocytic cells containing vacuoles and chromatin debris were prominent in the primary neoplasms (fig. 23). Leukemic cells were never recognized in the blood smears.

Summary of changes in the reticular system.—Changes were recognized after periods of 8 weeks or more following injection of the cell-free extract of leukemic spleens. The megakaryocytes in the bone marrow often contained one or more granulocytes; lymph nodes from 3 mice showed nests of hyperplastic reticulum cells in limited areas; the spleens were regularly increased in size and showed a cellular hyperplasia especially conspicuous in megakaryocytes, and the thymus showed a variety of changes. At an early period it was atrophic and the thymocytes were reduced; later, early leukemic changes appeared where a limited area contained large, basophilic, round cells interspersed with numerous macrophages. During this early stage, leukemic involvement of one lobe only was noted. Finally, widespread leukemia obscured the thymus and involved other organs. In only one leukemic mouse did the thymus appear not to be primarily involved, and in this mouse the thymus showed a peculiar granulomatous reaction.

Results of transplantation of splenic tissue.—Splenic tissue from each of 12 mice in the experimental group was transplanted to groups of 5 male BALB/c mice which ranged in age from 7 to 10 weeks (table 2). The first 5 transplants were intraperitoneal inoculations of 0.07 to 0.08 cc of cells. The spleens from the donor mice in this group were all greatly enlarged and came from mice in the 14- and 16-week periods in which the lymphocytic neoplasm involved many other organs. Leukemia arose rapidly in all the inoculated mice. In transplant #1, the mice appeared to be in poor condition 10 days after the transplant was made. They were autopsied, and the neoplastic cells were widely distributed in many organs and appeared in considerable numbers in the blood. The peripheral lymph nodes were enlarged. A distinctive feature of this transplant series, not observed in any other transplanted line, was the presence of masses of neoplastic cells within the adrenal medulla. Each of the other 4 intraperitoneal transplanted groups also showed a distinctive pattern with regard to the sites of localization of the neoplastic cells. In #2, the spermatic cord was a special site of leukemic infiltration; in #3, while the peripheral nodes were not enlarged, the blood smear showed many leukemic cells; in #4, the kidneys were heavily and diffusely infiltrated, and in #5, the intertubular tissue of the epididymis was infiltrated.

The remaining 7 transplants were made into a subcutaneous site from splenic tissue of supposedly leukemic mice. A small mass of splenic tissue was placed in a trocar and introduced beneath the skin of the back. The

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TABLE 2.—Spleen transplants—leukemic

Donors					Recipients							
							Spleen weight in grams		Leukemic organs			
Number of transplant	Weeks since injection	Spleen	Other organs	Mode of tranefer	Number of mice autopsied	Time to autopsy in days	Average	Range	Thymus	Peripheral nodes	Blood	Organ specially involved
1	14	Leukemic	Leukemic	Intra-peritoneal	4*	10	0. 81	0. 67–1. 00	2	4	4	Adrenal medulla
2	14	"	"	"	5	16	0. 75	0. 27–1. 28	4	2	0	Spermatic cord
3	14	"	"	"	5	16	0. 92	0. 57–1. 24	5	0	5	Peripheral blood
4	16	"	"	"	5	18	0. 74	0. 68–0. 87	5	5	4	Kidney
5	16	"	"	"	5	20	0. 57	0. 46–0. 64	5	5	4	Epididymis
6	20	"	"	Subcutaneous	5	31	0. 20	0. 16–0. 25	1	0	0	Extremely large local growth 1.5–4 cm diameter
7	21	"	"	"	4*	44	†		1	0	0	3 Large local growths, only 1 widespread neoplasm
8	20	0. 40‡	"§	"	4*	63	†		4	4	0	Small local growth in 3, no local growth in 1

* Transplant failed to grow in 1 mouse.
† Weights of spleen not taken. Grossly enlarged.
‡ Weight taken of spleen in donor mouse. Histologically no leukemic cells.
§ Thymus only.

results of the subcutaneous transplants from 3 leukemic spleens differed from the intraperitoneal route in showing a longer latent period. In transplants 6 and 7 a large local growth arose at the site of transfer with relatively little involvement of other organs. Transplant 8 is of special interest, because, although the thymus was leukemic, no neoplastic cells were recognized in the donor spleen on histologic examination, yet lymphocytic neoplasms arose in the recipient mice. The neoplastic cells in these mice were more diffusely spread than in mice receiving transplants from #6 or 7; very little local growth was found, yet the spleens of the 5 recipient mice from #8, the thymus glands, and the peripheral lymph nodes were leukemic. The neoplastic cells in this line were characterized by early dispersal, with relatively little tendency to form a localized growth.

Transplants 9, 10, 11, and 12 were made from enlarged spleens of mice in the 20- and 21-week group (table 3). Since the histologic sections from mice autopsied during the preceding weeks had not been reviewed at that time, it was supposed that the splenic enlargement was caused by infiltration with leukemic cells and that transfer of splenic tissue would produce leukemia in the recipient mice. In these 4 transplanted groups, however, no subcutaneous growths appeared and no generalized leukemia within the time expected after a neoplastic cell transfer. The mice were kept under observation, however, and after 13 to 15 weeks enlarged spleens were palpated. The mice were killed and at autopsy the remnants of the transplanted spleens were easily identified as small pigmented areas in the subcutaneous tissue on gross examination. Histologically splenic elements and many macrophages with hemosiderin were noted. The spleens of the mice in these 4 transplant groups were enlarged from 2 to 5 times the usual size, and there was cellular hyperplasia with an increase in megakaryocytes. The thymus showed atrophy, and megakaryocytes containing granulocytes appeared frequently in the bone marrow. One recipient mouse each in the 11th and 12th transfer groups developed a lymphocytic neoplasm still confined to the thymus glands at the time of autopsy. Thus the recipient mice receiving grafts from the nonleukemic spleens of virus-infected mice exhibited the same sequence of changes already described in the mice that received a cell-free extract from leukemic spleens, and in 2 of these mice a lymphocytic neoplasm confined to the thymus had developed. This assortment of lesions characterized the virus inoculation, and not a neoplastic cell transfer.

In a search for additional evidence that these induced lymphocytic neoplasms were not identical, the neoplastic cells from 6 original mice (3, 4, 5, 6, 7, and 8) and the cells in neoplasms arising after transfers from each of them were photographed at the same magnification. Although there was some variation in size of the cells from the various lines, it was too slight to be used as a distinguishing feature (figs. 26, 27, and 28), and no morphologic differences among the neoplasms could be shown.

TABLE 3.—Spleen transplants—nonleukemic histologically

Donors				Recipients							
Number of transplant	Weeks since injection	Spleen weight	Thymus	Mode of transfer	Number of mice	Weeks to autopsy	Spleen weights		Thymus		Mgk.*	Remarks
							Average	Range	Atrophy	Leukemia		
9	20	0. 39	Atrophy	Subcutaneous	5	15	0. 40	0. 37–0. 45	5	0	4	Microscopic hyperplasia of spleen†
10	21	0. 34	"	"	5	13	0. 28	0. 17–0. 37	4	1	3	Leukemia in thymus only in 1 mouse
11	21	0. 37	"	"	5	13	0. 25	0. 18–0. 40	4	1	3	"
12	21	0. 38	"	"	4	13	0. 29	0. 18–0. 32	4	0	3	"

*Granulocyte inclusions in megakaryocytes.
†This appeared in all mice from these 4 groups.

DISCUSSION

This experiment was designed primarily as a comparative morphologic study. We have endeavored to present an objective report of the differences observed in 2 groups of mice of the same inbred strain and of the same age and sex, when one group received a cell-free extract containing a leukemia-inducing agent obtained from the spleen of leukemic mice, and the other an extract of normal spleen. Untreated stock mice of the same strain, age, and sex were also carried as additional controls. We do not understand the significance of many changes observed and can only report that they were found in the experimental mice and not in the controls.

The organs in which a significant difference appeared all belonged to the reticuloendothelial or blood-forming system. The earliest difference found was the frequent appearance of one or more granulocytes within the cytoplasm of megakaryocytes in the bone marrow. If this is an artifact, it is curious that it was so frequent in the virus-treated mice and infrequent in the control group and stock mice. Only a few reports of this condition in megakaryocytes were located. A paper by Downey, Palmer, and Powell (*9*) described such megakaryocytes in a human atypical myelosis. The authors debated whether this was an attempt on the part of the neutrophil to ingest the megakaryocyte, or simple phagocytosis by the megakaryocytes. The rarity of erythrocyte ingestion was considered evidence against phagocytosis by the megakaryocyte. These granulocyte-containing megakaryocytes have also been seen in the bone marrow and spleens of mice ingesting 2-deoxyglucose (*10*) and in the bone marrow of dogs after sensitization with an antiplatelet serum (*11*). In a paper on the origin of megakaryocytes, Potter and Ward (*12*) photographed a megakaryocyte containing structures, which they thought might be granulocytes, in a mouse from the high leukemia strain C58. We have reviewed a few bone marrow sections from 1-year-old strain C58 mice and found that the granulocyte inclusions in megakaryocytes are much more frequent than in bone marrow sections from 1-year-old mice of strains A or DBA. Many virus particles were found in an electron microscope study of the megakaryocytes from mice infected with the Friend disease (*13*). No conclusion as to the significance of the megakaryocyte alteration can be drawn from this scant evidence, and certainly it is not pathognomonic of leukemia or a virus. More information is needed on the frequency of granulocyte-containing megakaryocytes, because they are easily overlooked unless specifically searched for.

In their study of the pathogenesis of leukemia in strain C58, Potter, Victor, and Ward (*1*) described a lesion in the lymph nodes which they thought was significant. This was a reticulum-cell hyperplasia with an associated lymphopoiesis, which originated in the medulla of the nodes. In terminal stages, evidence of the reticulum-cell participation disappeared. The lesion found in the lymph nodes of 3 mice in the present study was somewhat like the "Potter lesion," for there were nests of retic-

ulum cells interspersed with lymphocytes. In the 3 mice in which the lymph-node lesion was found, no other evidence of leukemia appeared. In the report by McEndy, Boon, and Furth (*3*), the lymph nodes were often involved early but no preneoplastic lesion was described. In the present study, the nodes were generally not uniformly and simultaneously involved; some might have been leukemic while others still appeared normal. Since lymph nodes are not satisfactory for comparative studies because of their morphologic variation, probably because of differences in activity, only extreme changes should be given consideration. Work to be reported later on the effects of this leukemia-inducing virus suggests that lymph-node involvement appears more frequently in thymectomized or splenectomized mice than in intact mice.

Undoubtedly the most clearly preneoplastic and neoplastic changes appeared in the thymus. These changes, however, were not uniform in morphologic features or time of appearance. Atrophy of the thymus with a reduction in thymocytes appeared after 8 weeks. The first leukemic changes in the thymus sometimes affected one lobe only. When the leukemia was fully established and obscured the early changes, the thymus area usually showed the most extreme involvement, and spread of the neoplasm usually appeared to extend from the thymus.

Origin of leukemia in the thymus gland has often been reported in the mouse (*5*), and a preneoplastic atrophy or involution has been described. A reduction in numbers of thymocytes regularly appears after irradiation and treatment with many carcinogens. It also appears after stress, treatment with cortisone, and starvation. Therefore, a preneoplastic significance cannot always be ascribed to this reduction, but thymic atrophy was a feature in the mice treated with the leukemic extract. The variation observed in 2 lobes of the thymus gland suggests that the organ did not respond as a unit to the treatment. Kaplan (*2*) observed that one lobe only might show the early changes of leukemia in C57BL mice after irradiation. The preleukemic changes described by Arnesen (*4*) in AKR/O mice were similar to those we observed in the virus-infected BALB/c mice in that the thymus glands in both studies showed a reduction in thymocytes in the cortex. The hyperplasia of epithelial reticulum described by Arnesen in his mice was not observed to the same degree in the virus-injected BALB/c mice.

One thymus was remarkable because it showed a peculiar granulomatous change, yet leukemic cells were not found in the thymus, though they were present in the other organs of this mouse. This was the only mouse in which it could be demonstrated that the leukemic change did not begin in the thymus. We had never before seen the nests of "foam" cells and acicular spaces, interpreted as cholesterol clefts, which characterized the lesion in the thymus gland of this mouse. Subsequently, however, a similar alteration has been found in the thymus glands of mice infected with this leukemia-inducing virus and later treated with X ray and with various chemotherapeutic drugs. At this time we can offer no suggestion regarding the significance of this granulomatous lesion.

All the neoplasms found in mice in this experiment have been diagnosed as lymphocytic neoplasms, since they were composed of mononuclear cells resembling lymphocytes. Experience with lymphocytic neoplasms in mice has shown that even though they may appear to be identical on morphologic study, the biological behavior of different lines on transplantation may reveal clear distinctions, as appeared to be true in this study. The opinion that nonidentical leukemias result from the action of this leukemia-inducing virus is supported by work to be reported later on the types of leukemia developing in virus-treated mice after thymectomy or splenectomy.

In the present study these neoplasms on transplantation showed differences in rapidity of growth, degree of cell dispersion, and organ involvement. We considered it important to prove, if possible, that different lines of lymphocytic neoplasms were induced, because this is evidence that the leukemias induced by the virus are not a simple cell transfer due to an error in technique, and that the virus acts like other carcinogens and does not mysteriously select a single cell and produce from it an invariable neoplasm of identical properties.

An unexpected finding was that transfer of a nonleukemic spleen from a virus-infected mouse would reproduce all the morphologic features of the virus disease and might even induce leukemia primary in the thymus gland; thus the same sequence of changes, which appeared after the injection of a cell-free extract, would be retraced. In one mouse, from which a spleen transfer was made, although the thymus gland was leukemic, histologic examination of the spleen failed to reveal malignant cells, yet leukemia developed in the recipient mice. This is an example of what McEndy, Boon, and Furth (*3*) called "the biologic test" of leukemia, that is, the successful transplantation from an organ in which malignant cells were not recognized microscopically.

Comparison of the two groups revealed no difference in any organs except the bone marrow, lymph nodes, spleen, and thymus. No endocrine disturbance was recognized by the methods employed, so that hormonal differences do not account for the high incidence of leukemia in the experimental mice. A precise and detailed study of the peripheral blood, which might have shown differences, was not made, but inspection of the blood smears indicated that no extreme variation would be revealed by a more intensive study.

No differences during the early days and weeks after the injection could be detected. In this respect, this leukemia-inducing virus resembles the milk agent that induces breast cancer in mice, for no differences have been found between mice with and without the milk agent in the months before the development of hyperplastic nodules and mammary tumors (*14*).

In the present study a few lesions found in the early period were equally frequent in mice inoculated with an extract of leukemic spleen, in those receiving injections of a normal spleen extract, and in the stock mice. Mice from all 3 groups showed an inflammatory reaction on the myocardial surface that has been previously described (*15*). Two

anomalies in the parathyroid glands were frequent. One was a small cyst lined by ciliated epithelium, similar to cysts frequently observed in the pituitary gland and the thymus of the mouse (*16*). The other was a mass of thymic tissue located in the parathyroid area, and previously described (*8*). A small lymphoid body was frequently found in these BALB/c mice. It was unlike either thymus or lymph node and we have found no previous description of it. The increase in mitotic figures and focal areas of necrosis found in the livers of mice from all 3 groups may indicate some infection affecting the liver in BALB/c mice. Since the lesions discussed were of equal frequency in all the mice examined, they are not considered significant to the outcome of the present experiment.

At the completion of any study and in retrospect, one usually becomes aware of how the procedure might have been improved. We regret that the weights of the spleen were not taken routinely, but we at first supposed that the enlargement was due to leukemic infiltration and that relative weights would have had little significance. We also regret that the thymus glands were not kept in separate bottles for histologic processing and that serial sections of the thymus were not done more often during the critical period when leukemia was developing. Peripheral blood counts, differential counts of the blood smears, and bone marrow smears would have been desirable, but would hardly have justified the labor involved when the outcome of the study was uncertain. Sectioning and study of the pituitary glands would also involve much labor and time, which could probably be more profitably used in a proposed study on rats in which leukemia can also be induced by this virus. Pituitary cells in the rat are more distinctive than in the mouse.

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PLATES

Photomicrographs are all from BALB/c mice started in experimental, control, or stock group when 6 weeks old. Time after start of experiment is given. All sections except one stained with hematoxylin and eosin.

PLATE 18

FIGURE 1.—Ectopic thymic tissue at site usually occupied by parathyroid gland. Control mouse, day 3. × 70

FIGURE 2.—Contiguous margins of lymph node on *left*, and lymphoid body on *right*. Lymphocytic cells in body are densely packed and appear larger and more basophilic than lymphocytes in lymph node. Marginal sinus in the body is crowded with cells. Control mouse, 15 weeks. × 210

FIGURE 3.—A lymphoid body next to submaxillary gland. *Note* absence of any separation into cortex with lymphatic nodules, and medulla. Sinus spaces are packed with intensely basophilic round cells. Edge of lymph node in *upper right*. Stock mouse, 11 weeks. × 100

FIGURE 4.—Liver with area of focal necrosis. Stock mouse, 11 weeks. × 100

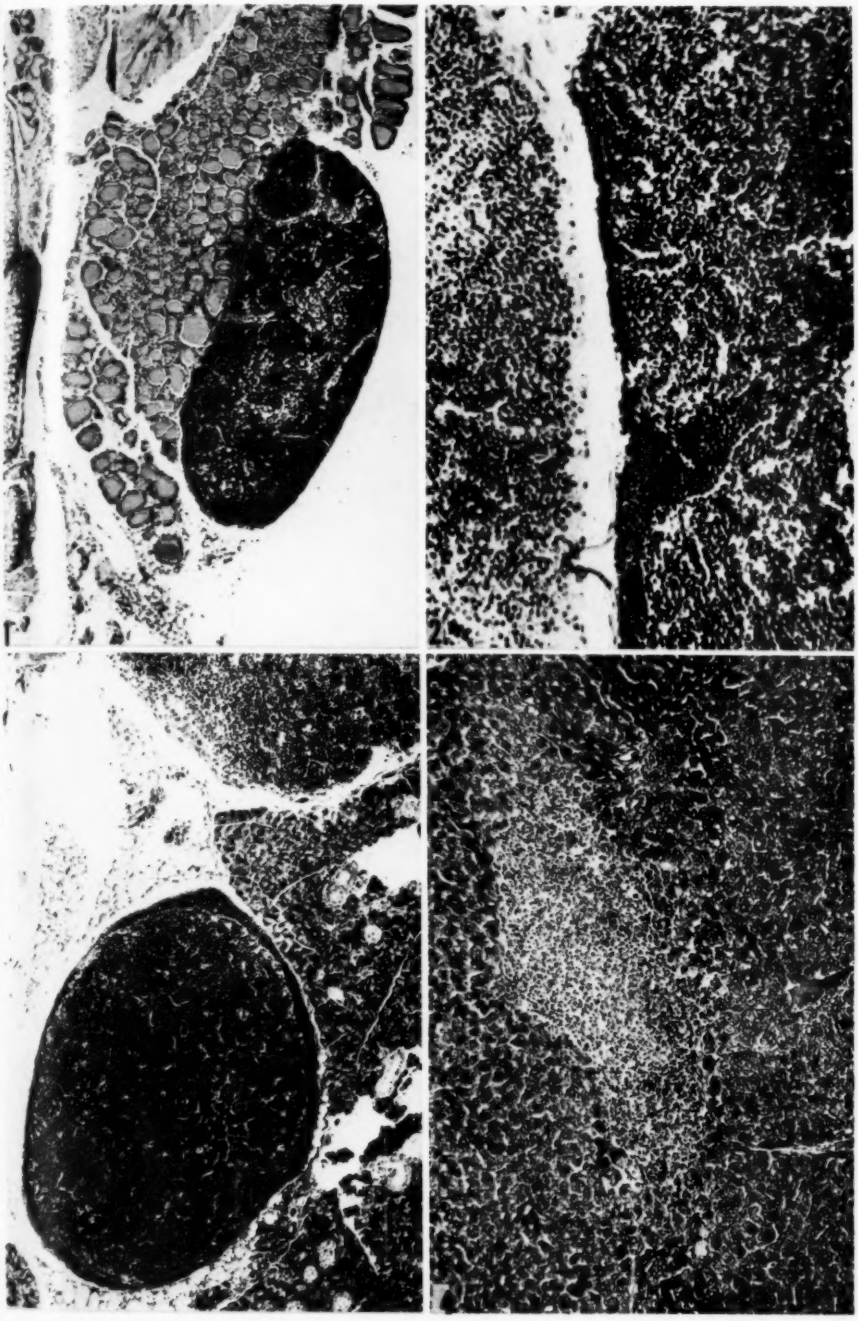

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PLATE 19

FIGURE 5.—Single pyknotic nucleus of granulocyte within cytoplasm of megakaryocyte in bone marrow. Narrow pale rim often surrounds granulocyte. Experimental mouse, 11 weeks. × 1,000

FIGURE 6.—Three granulocytes within megakaryocyte of bone marrow. Same mouse as in figure 5. × 1,000

FIGURE 7.—Single lobulated granulocyte within megakaryocyte. Experimental mouse, 20 weeks. × 1,000

FIGURE 8.—Three granulocytes within megakaryocyte. Mouse received tissue from nonleukemic spleen of virus-infected mouse 13 weeks before. × 1,000

FIGURE 9.—Higher magnification of granulocyte within megakaryocyte. Nuclei of contained granulocytes often appear pyknotic. Same mouse as in figure 8. × 1,500

FIGURE 10.—Granulocyte within megakaryocyte of spleen. Mouse received tissue from nonleukemic spleen of virus-infected mouse 15 weeks before. × 1,000

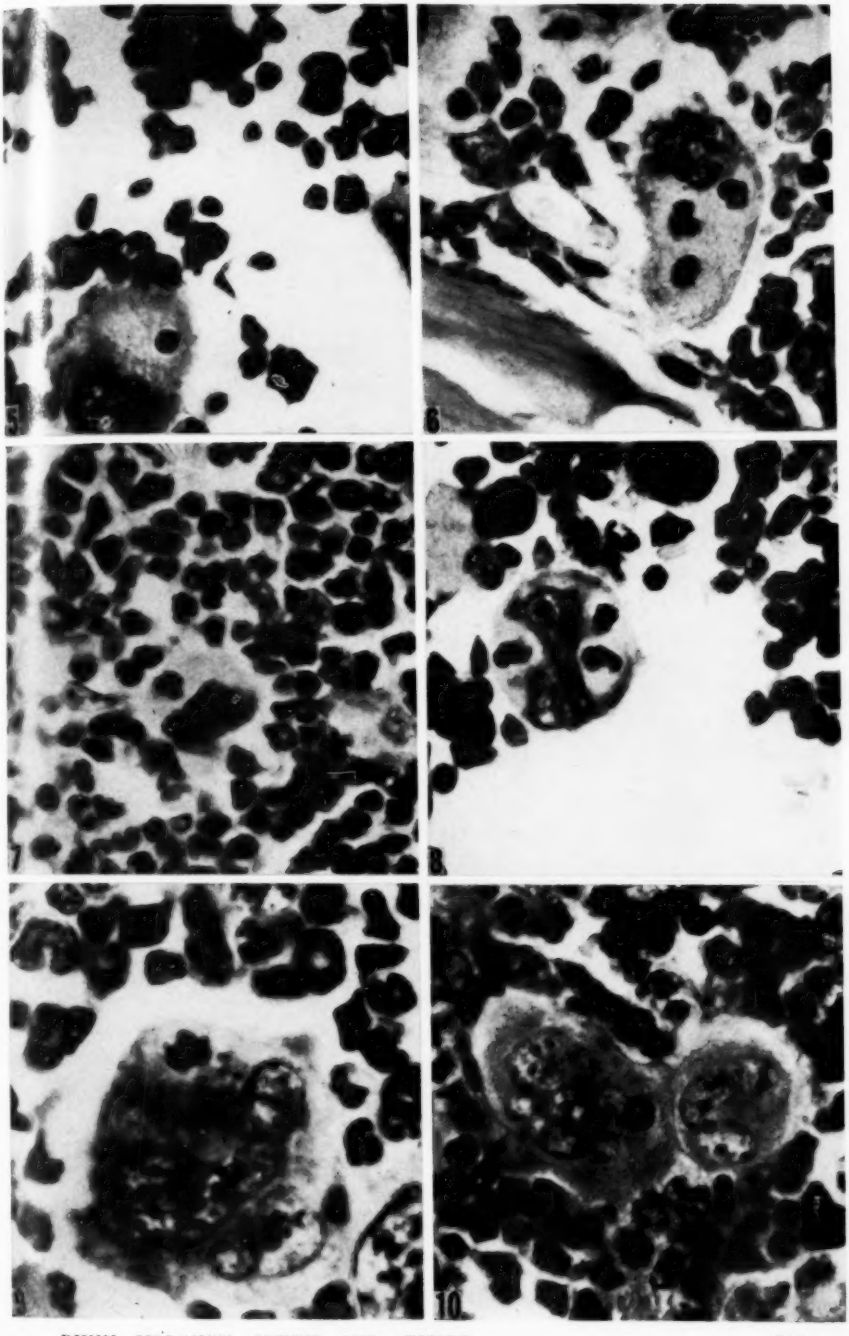


PLATE 20

FIGURE 11.—Mesenteric lymph node with circumscribed nodule in medulla containing focal areas of large reticulum cells. Normal lymphocytes appear between focal areas. Experimental mouse, 16 weeks. × 26

FIGURE 12.—Hyperplastic spleen, showing erythropoiesis and numerous megakaryocytes. Tip of spleen is rounded and area between nodules and capsule is wider than normal. Mouse received tissue from nonleukemic spleen of virus-infected mouse 15 weeks before. × 200

FIGURE 13.—Tip of spleen from control mouse showing fewer megakaryocytes than in spleen from virus-infected mouse. Control mouse, 5 weeks. × 200

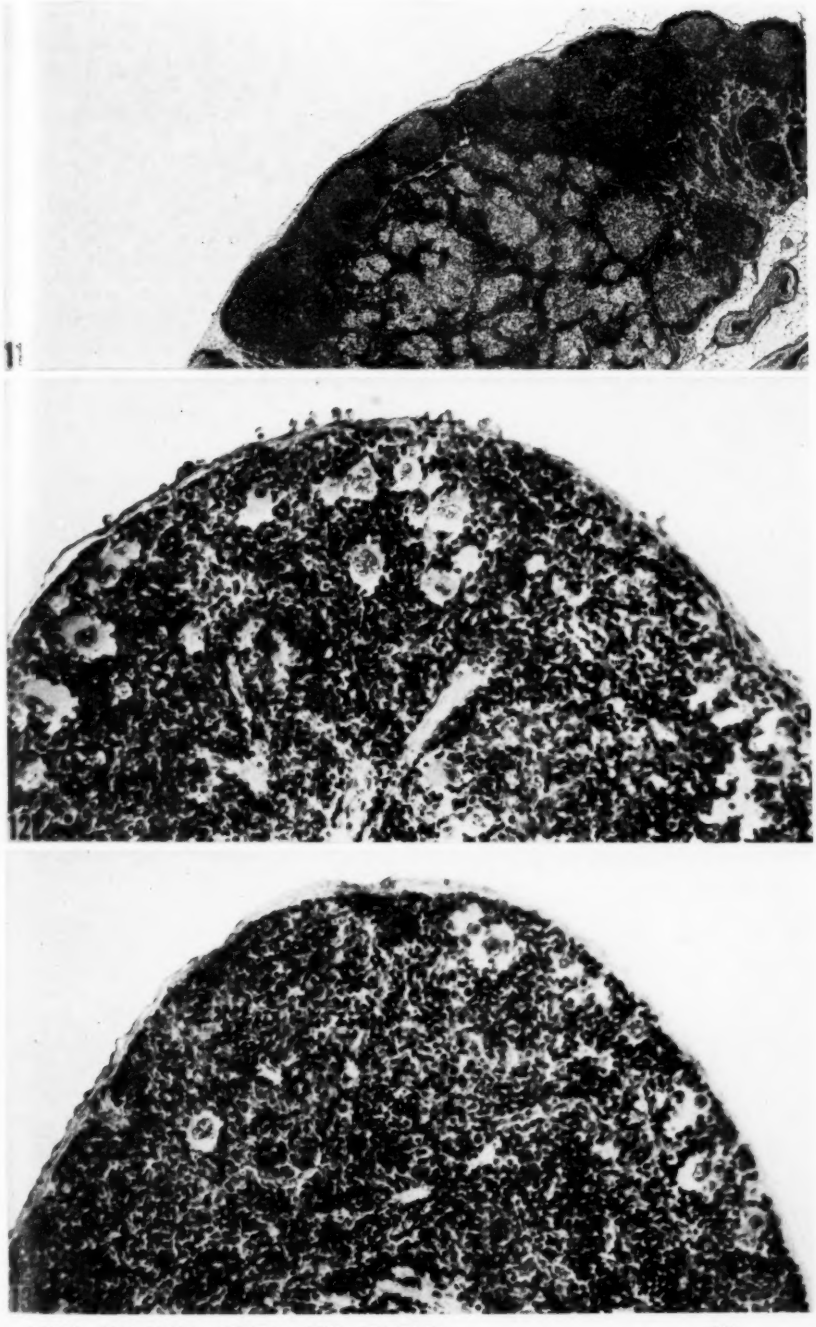


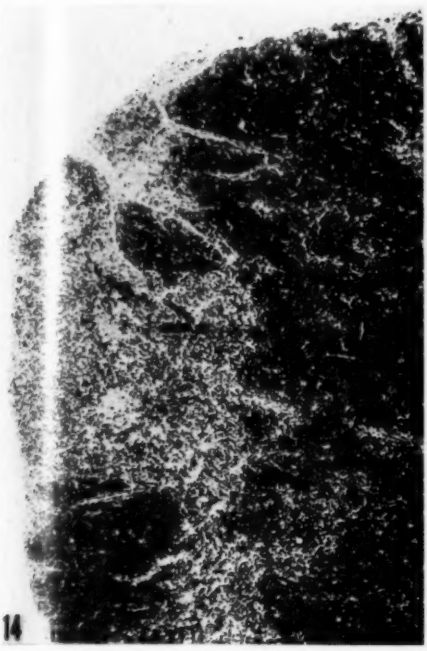
PLATE 21

FIGURE 14.—Thymus with medullary tissue composed of pale cells replacing are of cortex and extending to capsule. Experimental mouse, 16 weeks. × 90

FIGURE 15.—Thymic lobe on *right* shows numerous thymocytes in cortex. Lob on *left* is atrophic and contains few thymocytes. Lymphocytes are crowded int lymphatic at *lower right*. Experimental mouse, 14 weeks. × 90

FIGURE 16.—Thymus in hilus area and in lobe on *right* shows areas composed of larg foam cells with acicular clefts. In photograph, other lobe is atrophic, but in serial sections, foam cell areas were found at other levels in lobe on *left*. Small dark body in *upper left* is tracheobronchial node replaced by malignant lymphocytes. Experimental mouse, 14 weeks. × 24

FIGURE 17.—Higher magnification from same thymus shows foam cells and acicular clefts. Same mouse as in figure 16. Periodic-acid Schiff reaction. × 200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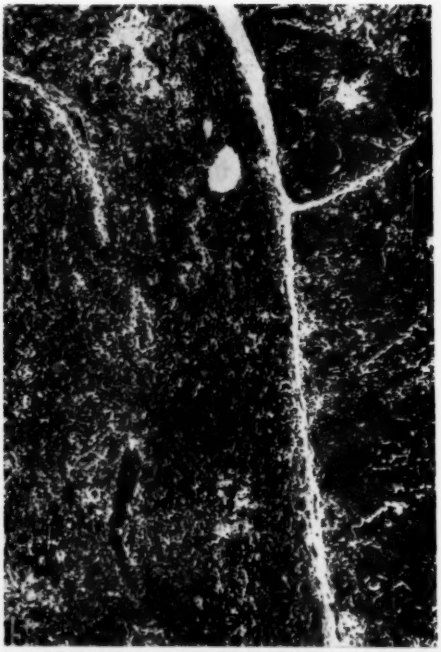
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PLATE 22

FIGURE 18.—Thymic lobe on *left* shows atrophy, with relative increase in width of medulla. Lobe on *right* shows early diffuse neoplastic change, with medullary tissue still visible. Experimental mouse, 11 weeks. × 28

FIGURE 19.—Thymic lobe on *left* shows atrophy with narrowing of cortex. Lobe on *right* shows leukemic cells interspersed with large macrophages. Experimental mouse, 13 weeks. × 20

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PLATE 23

FIGURE 20.—Thymus, tracheobronchial nodes, mediastinal adipose tissue and surface of heart are infiltrated with neoplastic cells. Experimental mouse, 21 weeks. × 8

FIGURE 21.—Thymus infiltrated with neoplastic cells. Darker central area with numerous macrophages probably represents previous outlines of thymus gland, while less basophilic area has developed beneath capsule. Experimental mouse, 16 weeks. × 90

FIGURE 22.—Small cluster of cells within leukemic thymus. Large, pale cells in this cluster, and elsewhere within gland, contained intranuclear eosinophilic masses surrounded by clear halo. These resemble nuclear inclusions but may be altered nucleoli. Eosinophilic bodies of this type were not found in any other experimental mouse. Experimental mouse, 15 weeks. × 1,500

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PLATE 24

FIGURE 23.—Neoplastic tissue in thymus composed of round cells with basophili nuclei and narrow rim of cytoplasm. Numerous pale macrophages containing nuclear debris are seen in this neoplasm. Experimental mouse, 16 weeks. × 1,000

FIGURE 24.—Cells from lymph node show characteristic cells with round or slightly indented nuclei, and narrow rim of cytoplasm. Experimental mouse, 21 weeks. × 1,500

FIGURE 25.—Cells in this neoplasm appear slightly larger and paler than preceding. Experimental mouse, 20 weeks. × 1,500

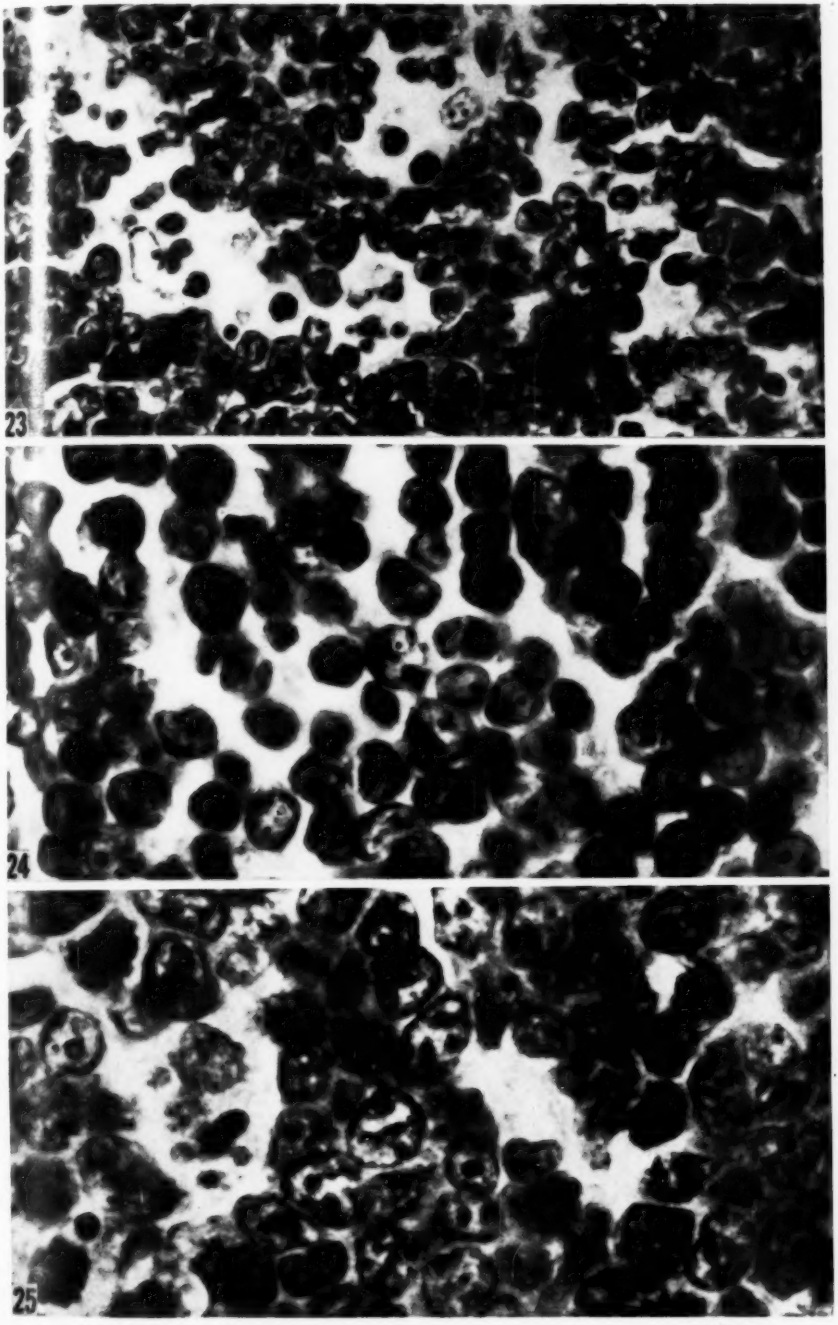
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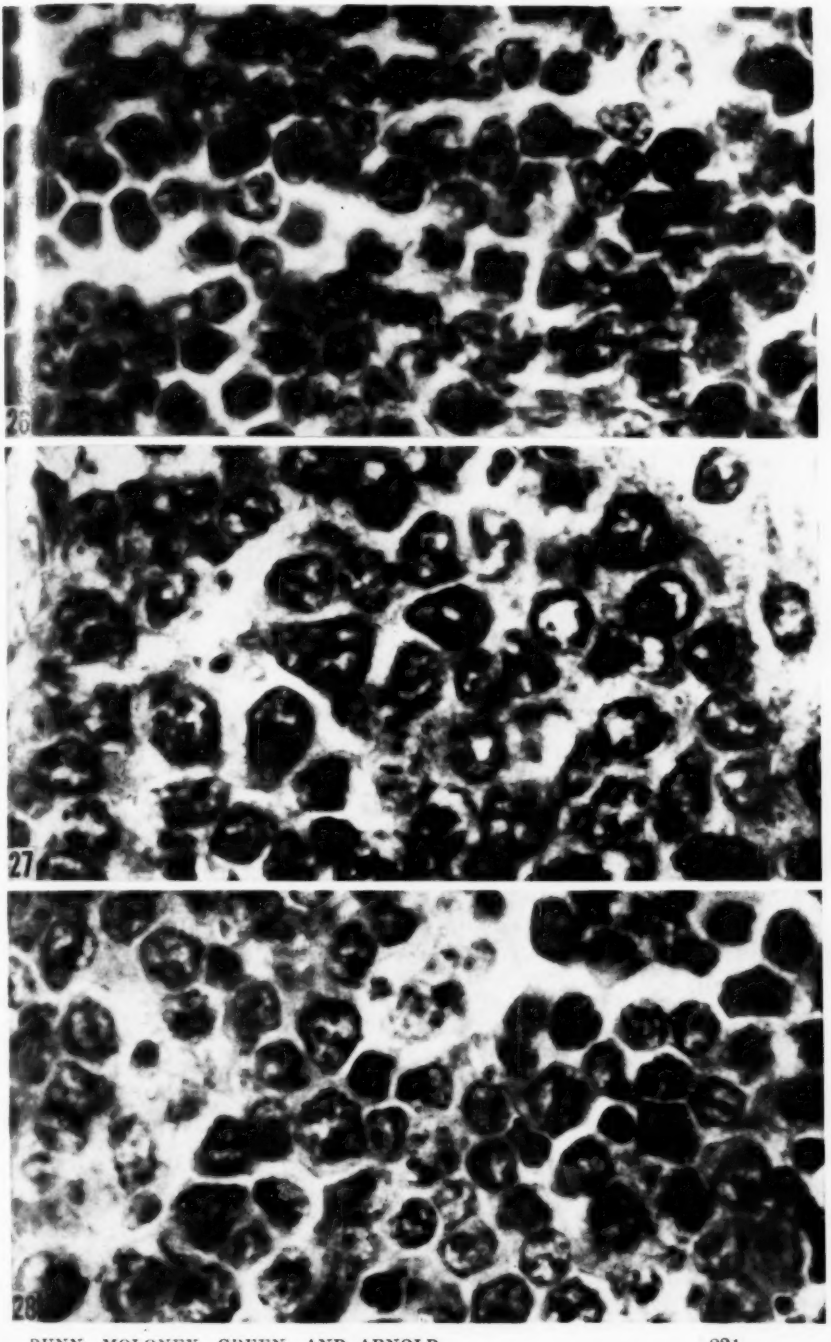
PLATE 25

FIGURE 26.—Usual large basophilic round cells composing lymphocytic neoplasm. Pale macrophage at *upper right*. Mouse received splenic tissue from experimental mouse in 20-week group 31 days previously. × 1,500

FIGURE 27.—Cells within this neoplasm appear larger, and nuclei are more basophilic than preceding. Mouse received splenic tissue from experimental mouse in 16-week group 20 days previously. × 1,500

FIGURE 28.—Cells appear paler and less regular in size than preceding. Mouse received splenic tissue from a mouse with leukemia in thymus 63 days previously. Transplanted spleen not leukemic on microscopic examination. × 1,500



Failure of Immunization Against Tumorigenesis [1, 2]

RICHMOND T. PREHN, *M.D., Department of Pathology, University of Washington, Seattle, Washington*

SUMMARY

Previous work from several laboratories established the existence of tumor-specific antigenicity in mouse sarcomas induced by polycyclic aromatic hydrocarbons. The possible existence of cross-reactions among various independently induced tumors provided the theoretical rationale for an attempt to immunize against carcinogenesis by exposure of mice to an antigenic tumor prior to exposure to the carcinogen. Fourteen trials involving a total of 20 different tumors were performed. Of 421 mice immunized, 267, or 63 percent became tumorous as a result of exposure to carcinogen. In a comparable period, 237, or 65 percent, of 375 unimmunized control mice developed tumors. Although one individual experiment apparently indicated immunization, the over-all results were completely negative.—J. Nat. Cancer Inst. 26:223–227, 1961.

THE WORK of several laboratories has demonstrated that most of the sarcomas induced in mice and rats by the polycyclic aromatic hydrocarbons are antigenic in the animal and in the inbred strain of origin (*1–6*). Much of this work has been controlled in a rigorous manner, and the fact of tumor-specific antigenicity in this type of neoplasm seems established beyond reasonable doubt.

As part of the earlier work in this laboratory, preliminary studies of immunologic cross-reactions were undertaken among various sarcomas induced independently by 3-methylcholanthrene (MCA). In one of three trials a cross-reaction was apparently demonstrated (*3*). This observation suggested the possibility of using tumor tissue to immunize mice against tumors yet to be induced—in effect, to immunize against carcinogenesis. This paper reports the results of experiments directed toward this goal.

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[2] This work was supported in part by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

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MATERIALS AND METHODS

All the experiments utilized the same basic plan with only minor variations. The mice used were of the inbred strains indicated in table 1; all had been raised in this laboratory and none of those used in any particular experiment was more than 4 generations removed from common ancestors. Their ages at the start of an experiment ranged from 2 to 5 months. The sexes are shown in the table. The animals were housed in plastic cages, approximately 8 to a cage, and given Purina laboratory chow and water *ad libitum*.

The fibrosarcomas used for immunization had been induced by the subcutaneous implantation of an approximately 2 mm diameter paraffin pellet containing 5 percent MCA. When used, the tumors were in the first to fifth transplant generation. Most of them had been demonstrated to be capable of producing immunity against the growth of implants of the same tumor in isogenetic mice. Some had been stored, prior to use, in a frozen tumor bank (*7*). In some experiments, as noted in the table, the immunizing tumor had originated in a mouse strain other than that in which it was employed.

Immunization was usually performed by the inoculation of the mice with a fragment of the particular tumor, with the use of a 13-gauge trocar. In experiment 2 a fragment of each of 3 different tumors was used in each mouse. In experiment 3 the mice were "reimmunized" 1 week after inoculation of the challenging carcinogen. In experiment 8, a cell suspension in saline solution incorporating cells of 9 different tumors was employed. The tumors were always placed subcutaneously (as superficially as possible) in the dorsal skin. When the fastest growing implant attained a diameter of about 10 mm (2 to 4 weeks), the implants were excised under pentobarbital-alcohol anesthesia (*8*) from all the animals, including any in which it might not have grown.[3]

Animals of control groups either were not inoculated, were inoculated with a fragment of normal liver tissue, or were inoculated with a nonantigenic mammary-tumor fragment. Control tissues were always isogenetic with respect to the mice in which the experimental tumor tissues had originated. Whether inoculated or not, control mice were subjected to an identical surgical procedure as were the immunized animals.

After removal of the immunizing tumor material, both immunized and control mice were implanted with carcinogen of the same type and in the same manner as that which had been used originally to produce the immunizing tumor. The carcinogen was placed, however, in the subcutaneous tissues of the ventral surfaces of the mice to avoid confusion with possible recurrences of the previously excised dorsal-tumor implants. In a recurrence of a dorsal-tumor implant, re-excision was done in both that animal and a matched control mouse. In 2 experiments the carcinogen was deposited in each of 2 separate sites in each animal.

[3] Many of these tumors grew poorly on initial transplantation despite genetic homogeneity among the mice (*5*). They seldom grew at all when implanted in a foreign strain.

TABLE 1.—Results of attempts to immunize mice against the occurrence of MCA-induced tumors

Experimental series								Control series						
Immunizing tumors				Experimental mice				Immunizing tissue			Control mice			
Experiment No.	Initiating carcinogen	Strain	Sex	Strain	Sex	Number of tumors/number of mice	Mean latent period	Tissue	Strain	Sex	Strain	Sex	Number of tumors/number of mice	Mean latent period
1	MCA	BALB/c	♂	BALB/c/An	♂	5/28	23	—	—	—	BALB/c	♂	16/28	19
2	MCA	BALB/c	Both	BALB/c/An	Both	8/17	20	—	—	—	BALB/c	Both	8/15	17.5
	MCA	C57BL	Both	BALB/c/An	Both	14/24	17.5							
3	MCA	C57BL	Both	C57BL/An	Both	6/14	13	—	—	—	C57BL	Both	7/16	21
	MCA	BALB/c	Both	C57BL/An	Both	12/25	21.5							
4	MCA	BALB/c	♀	BALB/c/An	♂	21/24	15	—	—	—	BALB/c	♂	19/23	18
				C3H/He		24/24	17.5				C3H		24/24	16
5	MCA	BALB/c	♀	BALB/c	Both	18/19	18	—	—	—	BALB/c	Both	12/19	16.5
				C3H/He	Both	17/20	18	—	—	—	C3H	Both	18/21	18
6	MCA	C3H	♀	C3H/He	Both	43/55	17	Breast tumor	C3H/He	♀	C3H	Both	36/42	15.5
				BALB/c/An	Both	41/50	15				BALB/c	Both	34/50	17
7	MCA	BALB/c	♂	BALB/c/An	♂	14/21	13	Liver	BALB/c	♂	BALB/c	♂	14/19	14
8	MCA	BALB/c	♂	BALB/c/An	Both	14/50	13	Liver	BALB/c	♂	BALB/c	Both	17/50	13
				C3H/He	Both	30/50	13				C3H	Both	32/50	13
Totals						267/421							237/357	
Percentages						63%							65%	

After implantation of the challenging dosage or dosages of carcinogen, the mice were observed at weekly intervals, and any resultant tumors were noted and an estimated average diameter recorded. Tumors were counted as having occurred whenever they reached an average diameter of 10 mm. The period of observation varied; in some cases the experiment was discontinued as soon as it was apparent that there would be no difference between the experimental and control groups. This fact accounts for much of the variation in mean latent period among the various experiments.

RESULTS

The results are presented in the table. It is obvious that the encouraging result of the first immunization trial was not duplicated in subsequent experiments. With the exception of the first experiment, none of the trials showed a convincing difference between the experimental and control groups, nor were the combined results impressive. Some minor differences that did occur were in the direction of apparently enhanced tumor formation rather than inhibition. Statistical analysis, while revealing increased variance among the experimental groups as compared with the control groups, indicated that this difference was not significant at the 5 percent level.

DISCUSSION

Whether the apparent success in the first experiment was due to a chance fluctuation or to a real immunizing effect cannot now be determined, because the particular tumor used is no longer available. It is possible that, because of differences in tumor antigenicity, an occasional tumor might be unusually effective.

Although the failure of these experiments, and those of Klein (*5*), to provide convincing evidence of immunity to carcinogenesis has been very discouraging, the negative results may mean only that the methods or techniques were inadequate. Perhaps the presence of the carcinogen itself blocked the immune response (*9*). However, the negative results do raise doubts concerning the assumption upon which the work was based—namely, the existence of immunologic cross-reactions among various MCA-induced tumors. Currently available data suggest that such cross-reactions may be unusual (*5*). If this is so, the theoretical rationale for these attempts at immunization against carcinogenesis does not exist.

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Bioassay on Polyvinylpyrrolidones With Limited Molecular Weight Range [1]

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SUMMARY

Two specially prepared polyvinylpyrrolidones (PVP's), one with a molecular weight range between about 2,000 and 38,000 and the other with a molecular weight range from approximately 4,000 to 80,000, were injected repeatedly as 25 percent solutions in physiologic saline into the abdominal cavities of rats and rabbits. The macromolecular material of both PVP's was retained in the organs of rats despite the relatively low maximal molecular weight of one of the PVP's. However, this PVP passed the glomerular filter of the rabbits and thus was not stored. The glomerular filter of rats and rabbits differed in pore size. The degree of storage of the PVP's tested did not exert any stimulating effect upon the production of cancers. In fact, there was evidence that one of two PVP's used for comparative purposes and having an average molecular weight of 50,000 might have interfered with the development of spontaneous cancers. Whenever carcinogenic properties of PVP's were present, they did not seem to be related to any physical properties of the macromolecules.—J. Nat. Cancer Inst. 26: 229–237, 1961.

PREVIOUS EXPERIMENTS with several polyvinylpyrrolidones (PVP's) of American, British, and German manufacture, having average molecular weights which ranged from 10,000 to 300,000, had shown that an appreciable fraction of such polymers dissolved in physiologic saline and parenterally injected was retained in various organs and tissues of mice, rats, and rabbits (*1*, *2*). The storage phenomena resulting therefrom resembled in degree, distribution, and histologic type those elicited by aqueous solutions of other water soluble synthetic and semisynthetic carbon polymers (*3*) in rats, rabbits, and dogs. These observations indicated that even PVP's of ordinary manufacture, having an average weight of 10,000, contain a fraction of sufficiently high molecular weight and size which cannot pass the glomerular filter or other biologic filter membranes and, therefore, cannot be excreted but is taken up by various phagocytic cells as well as cells of the parenchyma of several organs. Since some of the PVP's tested appeared to cause cancers in organs and

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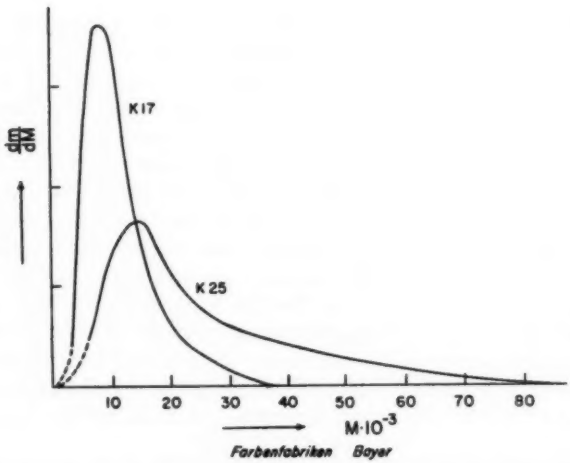
[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

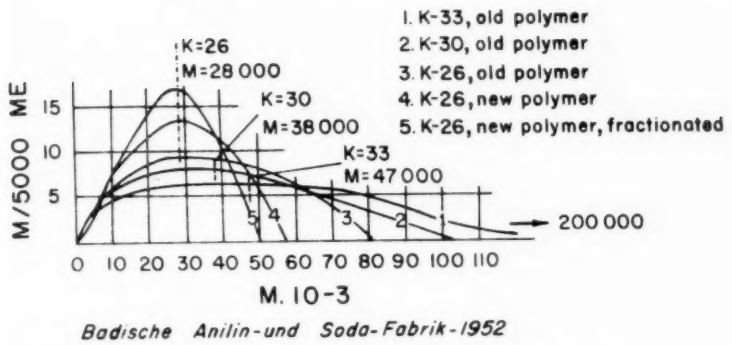
tissues in which they were retained, there was a possibility that a direct relation might exist between the molecular weight range, storage phenomena, and development of cancers by the PVP's tested. This concept could be put to a test when two PVP's of limited molecular weight range, prepared by the Badische Anilin-und Soda-Fabrik, Ludwigshafen, and furnished to me through the courtesy of Dr. Hecht, Farbenfabriken Bayer, were made available.

EXPERIMENTAL PROCEDURES

The two PVP's were furnished as 25 percent (w/v) neutral aqueous sterilized solutions in flasks, each containing 100 cc of a slightly yellow, viscous liquid. Kollidon K17 consisted of macromolecules having a molecular weight between approximately 2000 and 38,000, with the bulk of the molecules having weights between 5000 and 15,000 (peak at 10,000). Kollidon K25 was composed of molecules, the majority of which had a molecular weight of 15,000 to 30,000 (peak at 18,000), though an appreciable fraction had weights up to 80,000 (text-fig. 1). Active oxygen from the catalyst used was demonstrable in K17 to the amount of 0.05 percent expressed as hydrogen peroxide and in K25 to the amount of 0.005 percent. The molecular weight distribution of these two PVP preparations is identical with that found in newer types of kollidons (K26) manufactured by the Badische Anilin-und Soda-Fabrik, and differs distinctly from that of older preparations which displayed a distinctly larger spread of macromolecules into weights above 60,000, *i.e.*, of molecules which would not pass through the glomerular filter (text-fig. 2).



TEXT-FIGURE 1.—Molecular weight distribution of PVP's K17 and K25.



TEXT-FIGURE 2.—Molecular weight distribution of kollidon.

For comparative purposes, two PVP's (#6 and 7), one of American make and the second of German manufacture which had been used in a previous experiment in the form of subcutaneous implants of dry powder, were employed as 20 percent normal saline solutions. Both PVP's had an average molecular weight of 50,000. PVP #7 had displayed a weak carcinogenic effect upon repeated subcutaneous implantation.

With the use of sterilization techniques, the solutions of the 4 PVP's were administered at 2-week intervals by intraperitoneal injection into the abdominal cavities of 3-month-old female rats and rabbits weighing about 2500 g. To test the PVP's K17 and K25, groups of 35 rats of the NIH black strain were used. These received 4 injections of 2 cc containing a total of 2 g PVP. Groups of 6 rabbits of the Dutch strain received up to 5 injections of 50 cc containing a total of 62.2 g PVP.

PVP 6 (General Aniline and Film Corp., Plasdone C) was injected as a 20 percent solution in saline into 20 female NIH black rats, while PVP 7 (Schenley Laboratories, PVP PDR) was administered to 30 rats. Each rat was given 5 cc intraperitoneally at each of the injections, which were made 3 times a week for 5 weeks until 9 injections or 9 g PVP had been introduced into each rat. At this time, further administration was temporarily discontinued because of the animals' progressive and severe loss of weight. One month later, however, when the rats had recovered from the initial untoward effects, the injections were resumed until a total of 15 injections and 15 g—the amount originally planned—had been administered to each animal.

Two control rabbits received, in place of the PVP solutions, intra-abdominal injections with physiological saline in amounts of 50 cc each at intervals of 2 weeks and until a total of 250 cc had been given. Thirty NIH black rats likewise received intraperitoneal injections of 2 cc of normal saline (0.85% NaCl) for a total of 4 injections, given at intervals of 2 weeks.

Blood studies were performed on the rabbits which received PVP injections. The erythrocytes and leukocytes were counted in 2-week intervals before each injection. A differential count of the white cells was

made. After each injection the bleeding time, coagulation time, and sedimentation rate were determined at intervals of 1, 2, and 3 hours after each injection.

Rats surviving a maximal observation period of 2 years and rabbits after an observation period of 28 months, were killed. All animals were autopsied, and histologic examinations were made of all tissues which showed any macroscopic abnormalities. The sections were stained with hematoxylin and eosin.

EXPERIMENTAL OBSERVATIONS AND RESULTS

Table 1 presents the death distribution among the animals of the different series expressed in months of survival after the start of the experiment, as well as the number, sites, and types of cancers found.

TABLE 1.—Death distribution of rats and rabbits receiving injections of PVP, and number, sites, and types of cancers

Agent	Species	Number	Months of survival after start								Cancer (No.
			0–6	7–9	10–12	13–15	16–18	19–21	22–24	25–28	
PVP K17	Rat	35			3 1*	7 1*	9	4 1†	12 1†, 1*		5
	Rabbit	6			1				2	3	0
PVP K25	Rat	35	5	2	8	7	5	3 2†, 1‡	5		3
	Rabbit	6	2						1	3	0
PVP #6	Rat	20	5	1	3	1 1*, 1§	5	2	3		2
PVP #7	Rat	30	2	1	7	2	2	11	5		0
Control	Rat	30	2	1	5 1*	2	5	6 1†, 1*	9 2†		5
	Rabbit	2									0

*Reticulum-cell sarcoma of the liver.
†Carcinoma of the uterus.
‡Round-cell sarcoma of the ileocecal lymph nodes.
§Round-cell sarcoma of the subcutaneous tissue.

A prominent finding during the histologic examination of the tissues of rats and rabbits receiving injections of the 4 PVP's was the presence of light-blue-, deep-blue-, or slate-blue-stained homogeneous matter within foam cells as well as in the form of extracellularly located "lakes" in various tissues, such as the lung, heart, aorta, pulmonary artery, spleen, pancreas, choroid plexus, ovary, uterus, adrenal, liver, kidney, and stroma of some cancers (figs. 1 to 5). The organs and tissues of the control animals were totally free from such deposits, which were also absent in the tissues of the rabbits receiving solutions of PVP K17 but were well developed in those injected with PVP K25. In the rabbits receiving injections of PVP K17 solutions, the only histologically demonstrable findings suggesting that a transitory macromolecular colloidal

disturbance of the blood had occurred were the small, fibrous, intimal cushions in the aorta and in the branches of the pulmonary artery in 2 rabbits. The various types of cancers observed in the 4 series of animals receiving PVP's were identical in location and in structure with those seen in the control rats. In none of the test series were cancers more frequent than in the control series. In fact, cancers were not observed among the rats which received PVP #7.

The blood studies performed did not yield any marked acute or chronic effects of the colloidal macromolecular solution administered on the cellular constituents and the various properties of the plasma tested. Notably, there was no acceleration of the sedimentation rate of the erythrocytes within a 3-hour period after the intraperitoneal administration of the PVP solutions into rabbits. Several months after the start of the experiment the rabbits showed a moderate drop in the number of leukocytes, a shift toward a higher percentage of polynuclear leukocytes, and a moderate reduction in the number of erythrocytes and in the length of the coagulation and bleeding periods. The development of a characteristic hematic macromolecular syndrome (*3*) thus did not occur.

DISCUSSION

The observations made demonstrate that aqueous solutions of PVP K17 and K25 intra-abdominally injected into rats and rabbits were retained by rats like those prepared from PVP #6 and 7, despite the fact that at least the molecules present in PVP K17 did not surpass a molecular weight of 38,000 and could be expected, therefore, to pass through the glomerular filter of the rat kidney. It is remarkable, however, that blue-stained deposits were not demonstrable in the organs of rabbits receiving injections of PVP K17, which, therefore, presumably was readily excreted in the urine. This evident difference between the two species in the behavior of the permeability of the glomerular membrane to macromolecules present in the plasma might reflect a species-specific property. In this respect rabbits may resemble man more closely, while rats seem to have in contrast a glomerular membrane of lesser permeability or smaller pore size, if observations obtained by the use of globular macromolecules can be applied to linear molecules of similar molecular weight (*3*). The medical use of PVP's similar in molecular weight range to PVP K17, therefore, seems to have definite advantages over that of PVP's with a wide range of molecular weights.

The coexistence of the storage phenomena observed with a normal or even a subnormal cancer rate, the latter being present in rats given PVP #7, does not favor the view that a prolonged retention of PVP by itself in certain organs has any direct relation to the development of cancers attributable to PVP, unless the PVP used has carcinogenic properties inherent in the molecule or due to some unknown impurity. The observations made thus provide additional evidence indicating that

a number of physical properties of PVP's tested in these and previous experiments are unrelated to the carcinogenic properties exhibited by several types of these chemicals (*1*, *2*). In fact, the total absence of cancers among the rats receiving a solution of PVP #7 produces the impression that the massive storage of this PVP had hindered somehow the development of spontaneous cancer. This observation is similar to that previously reported by Stern *et al.* (*4*), who found that mice treated with PVP solution exhibited a reduction in the number of spontaneous breast cancers. Such a paradoxical behavior of PVP would be in line with the ambivalent carcinocidal-carcinogenic properties displayed by many applied physical and chemical agents, such as X radiation, radioactive substances, arsenicals, benzol, estrogens, urethan, sulfur and nitrogen mustards, and certain epoxides, applied in the therapy of cancer.

In view of the fact that the number of cancers observed in rats treated with PVP #2 also was unusually low, the probability exists that this apparent anticarcinogenic influence of massive administrations of PVP to the rats of these 2 series may not be attributable to any specific effect of the polymers, but might be accounted for by a marked interference with the general metabolism resulting from the presence of PVP in the cells of various organs, including the brain in the region of the third ventricle. The marked loss of weight of the rats of these 2 series observed during the period of PVP administration favors the concept of a nonspecific metabolic mechanism.

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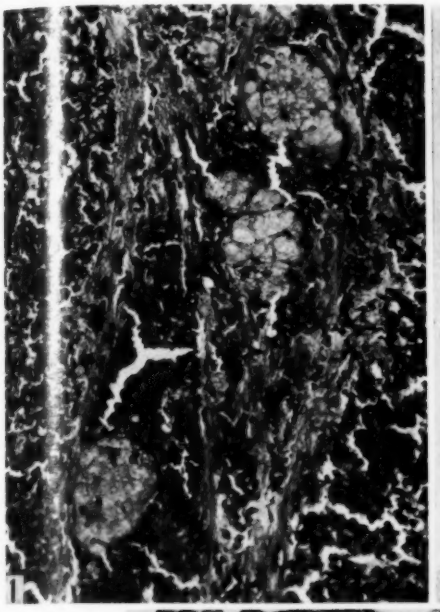
Plate 26

Figure 1.—Foam-cell clusters in the splenic pulp of a rat receiving injections of PVP K17. × 205

Figure 2.—Intra-alveolar foam-cell accumulations with a few multinucleated giant cells in the lung of a rat receiving injections of PVP K17. × 205

Figure 3.—Intra-alveolar foam-cell accumulation and interstitial foreign-body granuloma with giant cells in the lung of a rat receiving PVP #7. × 205



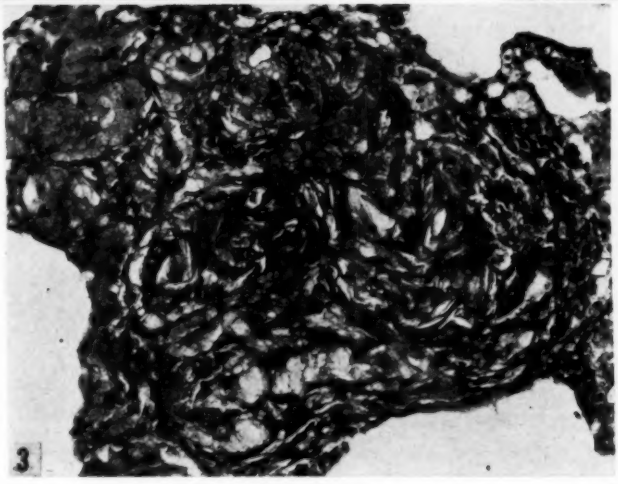
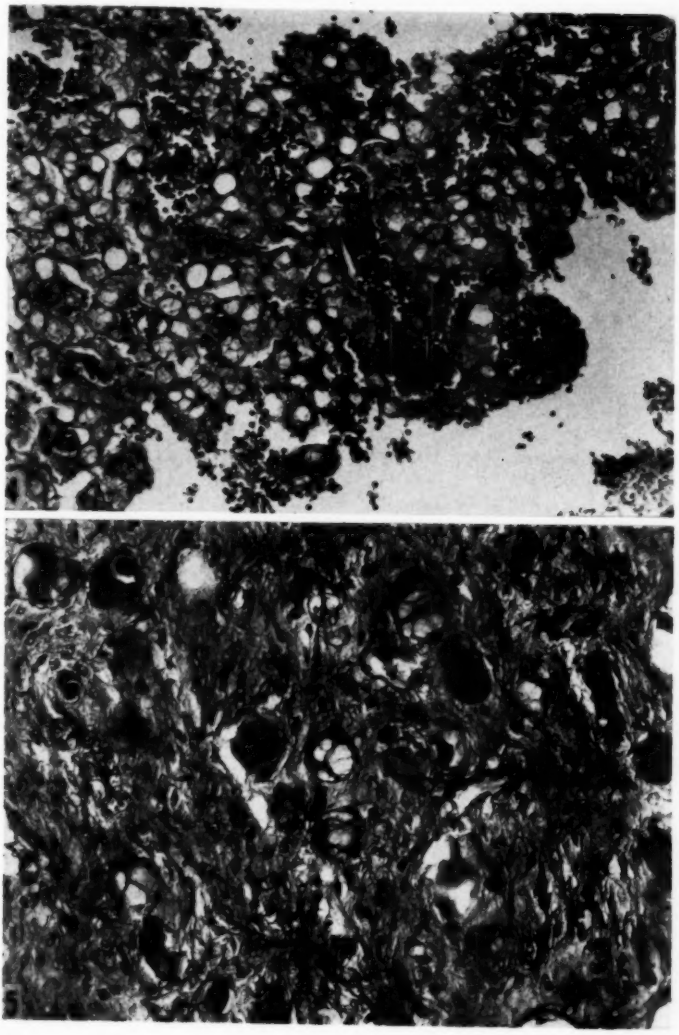


PLATE 27

FIGURE 4.—Foam-cell transformation of the cells of the choroid plexus in a rat receiving injections of PVP #7. × 205

FIGURE 5.—Deeply blue-stained foam cells in the hyaline endometrium of a rabbit receiving injections of PVP K25. × 205



Effect of Chemically Defined Medium NCTC 109 Supplemented With Methocel and of Silicone Coating the Flasks on Strain 2071 Cells in Suspension Cultures [1,2]

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SUMMARY

In earlier studies with rapidly agitated fluid-suspension cultures, cells of all strains tested on protein-free, chemically defined medium NCTC 109 had undergone rapid and complete disintegration. In the present study, this medium, supplemented with a methylcellulose, supported slow, logarithmic proliferation of cells of strain 2071 in such cultures, provided the inner walls of the flasks were coated with a silicone. Rates of increase ranging from 2.7 to 9.5 days were directly correlated with size of inoculum. Maximum populations attained at the end of the logarithmic phase in different experiments were about 1.1 × 10^6 cells/ml. Glucose was utilized at relatively low rates, 0.20 to 0.35 mg/10^6 cells/day. Methocel supplement in silicone-coated flasks was uniformly effective as a protective agent within the low viscosity grades and the wide range of concentrations tested. The roles of Methocel and of silicone are discussed, but explanation of their action must await further research.—J. Nat. Cancer Inst. 26: 239–252, 1961.

PROTEIN-FREE, chemically defined media, not supplemented with serum dialysate, protein hydrolysate, or antibiotics, have been used for serial propagation of mammalian cells in static cultures (*1–3*). In this laboratory, medium NCTC 109 (*1, 2, 4*) has proved fully adequate in static cultures for the continued proliferation of cells of mouse fibroblast strain NCTC 2071 (*5, 6*) and also of two other established strains (*7, 8*) for shorter terms. In rapidly agitated fluid-suspension cultures, medium NCTC 109 supplemented with horse serum or human serum at concentrations of 10 to 20 percent has given a more rapid rate of cell proliferation than has any other medium used with the cell strains tested (*9*).

In such rapidly agitated fluid-suspension cultures it had not been possible to dispense with serum supplement (*9*). Cells of all established strains, including NCTC 2071, in protein-free medium invariably underwent rapid disintegration. Since serum is a chemically undefined and

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[2] Much of this work was originally reported at the annual meeting of the Tissue Culture Association at Atlantic City, N.J., April 9, 1959.

[3] The authors wish to express their gratitude to Mrs. Evelyn Peake and Dr. W. C. Alford, Microanalytical Laboratory, Laboratory of Chemistry, National Institute of Arthritic and Metabolic Diseases, who performed the chemical analyses.

[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

complex natural product, a chemically defined nonprotein polymer high in molecular weight was sought as a substitute for the serum. An exploratory experiment along this line, reported previously (*9*), indicated that supplementation of medium NCTC 109 by the cellulose polymer Methocel of high viscosity grade, 4000 centipoises (cps), for 11 days at 0.1 percent concentration allowed maintenance of clone 929 cells at approximately the inoculum population. The cells, however, showed progressive, qualitative abnormalities. These results were interpreted to indicate that methylcellulose did not effectively protect against stress in the type of agitated fluid-suspension cultures used. Later, experiments of Merchant [5] with clone 929 cells in agitated fluid-suspension cultures indicated that low concentrations of Methocel of 15 cps viscosity grade, together with Difco-peptone (0.5%), effectively supplemented Morgan's chemically defined medium 199 (*10*) in promoting growth of these cells as evenly dispersed agitated suspensions. Kuchler, Marlowe, and Merchant (*11*) have carried clone 929 cells in agitated cultures on medium 199P (199 + 0.5% Difco-peptone), supplemented with Methocel, continuously for more than 2 years, with rapid rates of growth equal to those shown during the same period by cells of the same strain in the same type of culture on medium 199 supplemented with serum. Thus these workers have found the inclusion of a chemically undefined mixture of polypeptides and amino acids necessary as a supplement. The first report from Merchant led us to study more closely the use of Methocel of low viscosity grades as a supplement to chemically defined medium NCTC 109 in agitated fluid-suspension cultures.

MATERIALS AND METHODS

The type of agitated fluid-suspension culture and the procedures used for subculturing, fluid change, and nuclei count have been described (*17–20*). The first-generation cultures in each experiment were planted with cells from static flasks. For initial experiments special 25 ml roller tubes (*21*) were used on drums rotated at speeds of 1080 and 2160 rph; each tube contained 3 ml fluid suspension. In later experiments, flasks of 3 sizes were used: 250 ml flat-bottom boiling flasks containing 30 to 40 ml fluid, 500 ml boiling flasks containing 100 to 200 ml fluid, and 1500 ml flasks (*17*) containing 100 to 400 ml fluid. The flasks were agitated on the Brunswick shaker (rotary action flask shaker, model V; New Brunswick Scientific Co., New Brunswick, N.J.) at 8640 rph.

Two methods of aerating cultures and two kinds of gas mixtures were used. The roller-tube cultures and those in the smaller flasks were gassed only at planting and fluid change, with a mixture of 5 percent CO_2 and 95 percent air. The cultures in the 1500 and 500 ml flasks were aerated continuously with a mixture of 5 percent CO_2, 25 percent oxygen, and 70 percent nitrogen.

[5] Personal communication, April 9, 1958.

Selection of roller tubes for the exploratory experiments was based on our experience that, at the velocities used, cells in roller-tube cultures are much less subject to mechanical injury than in cultures in the larger flasks agitated on the Brunswick shaker.

Protective agents, such as polyvinylpyrrolidone (PVP), Methocel, dextrans, and alginic acid, were used by Katsuta *et al.* (*12–14*) for cells on protein-free medium. This medium was supplemented, however, with chemically undefined lactalbumin hydrolysate, and the cultures were in static flasks. Phillips and Andrews (*15*) have used methylcellulose as a protective agent for cells on balanced saline in static culture. Neumann and Tytell (*16*) found that Walker carcinosarcoma 256 cells grew well in suspension cultures on a medium without serum that contained, however, both insulin (now chemically defined) and lactalysate.

The present paper describes the clearly protective effect that low concentrations of Methocel of low viscosity grades have exerted on rapidly agitated fluid-suspension cultures of strain 2071 cells in protein-free chemically defined medium NCTC 109 not supplemented with serum dialysate or lactalbumin hydrolysate. Methocel is most effective, however, only if the inner walls of the agitated culture flasks have been coated with silicone.

Methocel supplement.—Methocel is the trade name for a group of cellulose polymers manufactured by Dow Chemical Company, Midland, Michigan. The approximate molecular weight range is from 19,000 to 150,000. (Information supplied by Dow Chemical Co.) The various types of Methocel have different proportions of certain substituents attached to the cellulose chain. Two types of Methocel were used: Methocel type 65HG, viscosity grade 50 cps, has 27 to 29 percent methoxyl, 5.5 to 7.5 percent hydroxypropoxyl substituents, and a molecular weight range from 46,000 to 50,000. Methocel type MC, grade USP, has 27.5 to 32 percent methoxyl substituents, a range giving maximum water solubility. The latter type was used throughout most of the experiments in 4 viscosity grades: 10, 15, 25, and 50 cps, over the molecular weight range from 19,000 to 50,000. The Methocels used were prepared in aqueous solutions that were sterilized in the autoclave. These sterile solutions were combined with equal volumes of 2× concentrated Earle's saline; the pooled solutions were added to medium NCTC 109 in such proportions as to give the desired Methocel concentrations.

Preparation of silicone-coated culture flasks.—In later experiments, the culture flasks were coated with silicone on the inner walls. Dow-Corning DC-200 (polydimethyl siloxane), viscosity grade 1000 centistokes at 25° C, was prepared as either a 5 or 10 percent solution in reagent benzene; this solution was flushed over the inside of the flask, which was then allowed to drain for 15 minutes. The flask was dried at 85° C for 30 minutes and was then baked at about 250° C for 3 hours. After cooling, the flask was filled with warm, triple-distilled water, allowed to stand for 2 hours, emptied, rinsed three times with triple-distilled water, and dried at about 100° C in a steam-jacketed autoclave chamber. The

flask throats were temporarily capped with aluminum foil that had been rinsed with acetone to remove any oil coating. (More recently, the special aluminum foil furnished by the manufacturer, Reynold's Metals, for general use in this laboratory is free from oil coating since it has been temper dry-annealed; therefore the acetone rinse is no longer necessary.) The flask was then sterilized in a dry-heat sterilizer for 3 hours at 170° C.

EXPERIMENTS

Significant experiments are presented in the chronological order in which the work developed. [The experiment number code relating the number used with the laboratory code numbers is: 1) 3103; 2) 3124; 3) 3150; 4) 3153; 5) 3180; 6) 3516; 7) 3687.]

Exploratory Experiments With Roller Tubes

Experiment 1.—Strain 2071 cells proliferated reasonably well in medium NCTC 109 in special roller tubes. The results from experiment 1 are presented as part of table 1. It is noteworthy that the control cultures (A) in unsupplemented medium showed some proliferation in view of the relative fragility of these cells. Proliferation rates of both groups of roller-tube cultures were low compared with rates previously reported (*18, 22*) for clone 929 cells in both large shaker flasks and in roller tubes in medium NCTC 109 supplemented with serum.

In these roller-tube cultures the presence of low viscosity Methocel in the medium consistently favored the attachment of some of the cells as a ring on the shoulder and as a sheet on the conical base of the special roller-tube flask. There were usually more cells adherent than in suspension; the closely massed adherent cells often became necrotic from overcrowding.

Experiments With Methocel Supplement in Agitated Flasks

Experiment 2.—The possibility of extrapolating the results from the roller-tube culture experiments to agitated fluid-suspension cultures was examined. During the first 12 days, three cultures in 250 ml flat-bottom boiling flasks were used for each treatment. Methocel of 50 cps viscosity graded supplemented NCTC 109 at 0.12 percent concentration in two treatments. Cultures on such Methocel-supplemented medium remained in healthy condition despite the lack of proliferation, while the control cultures on medium NCTC 109 alone disintegrated rapidly and completely. The experimental objective was therefore modified, at the 12th day, from a short-term quantitative test of several variables to a longevity test of the surviving Methocel-supplemented cultures. Two larger cultures were planted in 1½ liter flasks from pooled suspensions of the parent cultures. One of these cultures was carried for 7 weeks; the cells in both cultures remained normal in appearance, but proliferated very little. Both daughter cultures not only had many cells in suspension but also were charac-

TABLE 1.—Summary of pertinent data from major experiments with strain 2071 cells in medium NCTC 109

	Experimental conditions							Supplement to medium NCTC 109: Methocel			Population ($\times 10^6$) (*per culture*)		Logarithmic phase		
		Culture flask				Fluid volume per culture									
Expt. No.	Treatment	Number of cultures	Type	Silicone coat	Duration (*days*)	Initial (*ml*)	Final (*ml*)	Viscosity grade (*cps*)	Concentration (*percent*)	Other	Inoculum	Maximum	Population at end of phase	Duration phase (*days*)	Generation time (*days*)
1	A	6	Roller tube	−	10	3	3	—	—	—	1.6	3.4	3.4	10	8.80
	B	6	Roller tube	−	10	3	3	50	0.12	—	1.6	4.1	4.1	10	7.17
4	A	2	250 ml	+	1	30	40	—	—	—	17.2	17.2	—	—	—
	B	2	250 ml	+	12	30	40	—	—	Serum	17.2	76.3	62.4	5	2.68
	C	2	250 ml	+	12	30	40	15	0.06	—	17.2	67.0	42.7	5	3.80
	D	2	250 ml	+	12	30	40	15	0.12	—	17.2	69.0	42.9	5	3.78
	E	2	250 ml	+	12	30	40	15	0.06	—	17.2	69.4	43.5	5	3.73
	F	2	250 ml	+	14	30	50	15	0.12	—	17.2	88.5	32.2	5	5.50
	G	2	250 ml	−	9	30	40	15	0.06	—	17.2	26.0	26.0	5	8.33
5	A	1	1.5 liter	+	21	100	400	10	0.06	—	94.7	784.0	784.0	21	6.88
	B	1	1.5 liter	+	12	100	200	10	0.12	—	106.2	184.2	184.2	9	11.34
	C	1	1.5 liter	+	21	100	400	15	0.06	—	110.7	452.0	452.0	21	10.34
	D	1	1.5 liter	+	21	100	400	15	0.12	—	89.7	482.6	482.6	19	7.82
	E	1	1.5 liter	+	21	100	400	25	0.06	—	98.4	368.0	368.0	21	11.03
	F	1	1.5 liter	+	21	100	400	25	0.12	—	107.4	325.3	325.3	19	11.88
	G	1	1.5 liter	+	12	100	100	—	—	Serum	89.7	89.7	—	—	—
6	1	1	1.5 liter	+	21	100	400	25	0.12	—	40.4	530.5	455.3	17	4.86
	2	1	1.5 liter	+	17	100	400	25	0.12	—	31.7	392.7	392.7	17	4.68
7	1	1	500 ml	+	13	100	200	10	1.02	—	38.0	141.8	141.8	13	6.84
	2	1	500 ml	+	13	100	200	50	0.44	—	47.2	175.6	175.6	13	6.86

terized by 1) adhesion of cells to the sidewalls in a continuous ring at wave crest,[6] 2) formation of a uniform sheet of cells on the floor of the flask, and 3) the presence of two distinct types of nuclei in the stained preparations used for nuclei counts. The first two of these conditions were inhibitory to optimum development of a fluid-suspension culture; therefore the wavecrest ring and other adherent cells were removed from the glass and resuspended in the fluid suspension before each nuclei count and fluid change. We accomplished removal by gently brushing a small piece of sterile, perforated cellophane across the area, which thus minimized the possibility of cell damage. Two distinct kinds of stained nuclei consequently appeared in the hemocytometer chamber at nuclei count: (*a*) normal, large nuclei with well-defined chromatin networks and clearly seen nuclear membrane, and (*b*) smaller nuclei with a heavily stained nuclear membrane and a poorly expressed chromatin network which suggested cell disintegration. We checked the origin of the two kinds of nuclei by taking aliquot samples of suspension for nuclei counts immediately before the cells in the wavecrest ring were displaced into the suspension and again just after. Only normal nuclei were found in the first sample; both normal nuclei and many small nuclei in the second.

Experiment 3.—Since Methocel, 50 cps at 0.12 percent, was effective in protecting cells, further experiments were carried out to confirm this and to examine the effect of some other low viscosities and concentrations. Two concentrations (0.06 and 0.12%) of the 50 cps viscosity grade and one concentration (0.12%) of the 15 cps viscosity grade were used. Fluid change protocol, size of culture, and replication per culture were the same as in the preceding experiment: 30 ml of suspension/250 ml flask during the first 12 days, then pooling and subculturing into one 100 ml culture in a 1½ liter flask.

The 30 ml cultures proliferated slowly to the 8th day; the population then leveled off into a stationary phase, which persisted to the 30th day. After that, the population of the cultures declined and the experiment was closed on the 35th day. All Methocel treatments gave similar proliferation rates.

A quantitative estimate of the relative numbers of cells attached in the ring, as well as of cells in free suspension in the 100 ml cultures, was made on the 16th day. As in the preceding experiment, samples for nuclei count were taken from each of two cultures, first, before the cells in the ring were removed and resuspended, and second, just afterward at the usual time and condition of sampling. The ratios between the number of cells contained in the ring and the number of free-floating cells in the culture suspension were 0.42 to 1 and 0.31 to 1, respectively, for the two cultures.

These experiments demonstrated that low viscosity Methocel adjunct

[6] When a large flask culture is being agitated on the Brunswick rotary shaker, the surface of the liquid in the flask assumes a complex, generally concave pattern. At the flask wall a wave of nearly constant amplitude travels around the flask. The high point of the wave, designated the wave crest, describes a circle at about constant height above the floor of the flask. It is in this wavecrest zone that cells tend to mass and adhere to the glass.

to medium NCTC 109 enabled cultures of the 2071 strain of cells to proliferate slowly, and then to remain in a stationary phase for more than 2 weeks. Reduction or prevention of the accumulation of a relatively large fraction of the total cell population in the wavecrest ring zone appeared to be essential in further work.

Experiments With Methocel Supplement and Silicone Coating in Agitated Cultures

Experiment 4.—The 15 cps viscosity grade of Methocel was used because in previous experiments the protective effect of this grade was fully equal to that of 50 cps Methocel. The major objective was to test the effectiveness of an extremely thin, hydrophobic coating on the inner walls of the culture flask in preventing the formation of a wavecrest ring of cells. Silicone was used because of its easy applicability, inertness, and generally nontoxic nature.

Seven variables with two cultures each were tested. For the first 7 days each culture (in a 250 ml flask) was maintained at a volume of 30 ml; between the 7th and the 12th days the volume of each culture was 40 ml. On the 12th day the 2 cultures in each of the 5 variables then remaining were pooled and subcultured in 1½ liter siliconed flasks, each with 100 ml culture fluid.

Population trends during the first 12 days are summarized in table 1 as average values per culture in each treatment. All the cultures receiving a supplement to medium NCTC 109 proliferated at a moderate rate; average generation times during the first 5 days were 2.7 days for the pair of cultures receiving serum supplement (B), 4.1 days for the 4 pairs of cultures receiving Methocel supplement (C, D, E, and F), and 8.3 days for the pair of cultures receiving Methocel, but in a nonsiliconed flask (G). Cells of the pair of negative control cultures (A) that received only NCTC 109 without either serum supplement or Methocel had completely disintegrated by the 2d day of the experiment, even though the cultures were in siliconed flasks.

During the second generation, not shown in table 1, the 4 cultures receiving Methocel supplement (C, D, E, and F) remained in the plateau phase for different periods, up to the 30th day of the experiment. The population of culture B receiving serum supplement, however, declined precipitously for several days before the cells completely disintegrated.

It was concluded that both the Methocel supplement and the silicone coating of the flask walls, within the ranges tested, seem to be necessary for optimum proliferation and culture conditions. In silicone-coated flasks, Methocel was nearly as effective as serum in protecting the cells during the logarithmic phase and initial plateau phases; later in the life of the culture Methocel was clearly more effective than serum, since the culture receiving serum deteriorated.

Experiment 5.—The objective was determination of the relative effect of three low viscosity grades of Methocel. Seven different treatments

were each represented by one large culture in a standard 1½ liter shaker flask, coated on the inner walls with silicone.

The first culture received medium NCTC 109 supplemented with 20 percent horse serum and without Methocel. The culture deteriorated until it was discarded on the 9th day. The 6 remaining cultures received medium NCTC 109 supplemented with Methocel of 3 viscosity grades, 10, 15, and 25 cps and at 2 concentrations. These cultures all proliferated. From an average inoculum of approximately 100 million cells in 100 ml, these cultures proliferated slowly but steadily during the first 21 days. Average generation time was 9.45 days, a slow rate compared to rates previously reported for agitated cultures of the same cell strain in non-siliconed flasks on medium NCTC 109 with 20 percent horse serum (*18*). On the 21st day the cell populations averaged 472×10^6/400 ml culture and the cell concentrations averaged 1.2×10^6/ml. The volume of fluid in each culture had been increased gradually at each fluid change until the 14th day to a maximum volume of 400 ml, a level maintained during the remainder of the experiment.

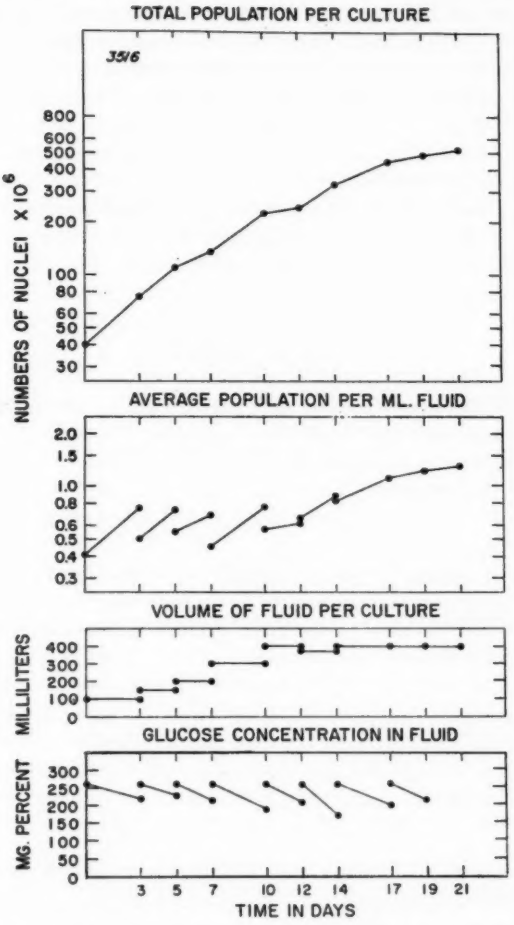
The daughter cultures from subculturing on day 21, not represented in table 1, had less regular proliferation than those of the parent cultures. The average population increased slowly from 185×10^6 on day 21 to 550×10^6 on day 63; culture volumes were uniform at 400 ml.

In all Methocel-supplemented cultures the cells in suspension and the stained nuclei in the hemocytometer chambers appeared normal. Gradual erosion of the silicone coating in a belt about 1 cm wide at the wavecrest zone was observed in some flasks. Occasional free-floating silicone flakes of about 100 μ diameter were observed, none of which were attached to the cells; these flakes came from narrow, crazed belts on the walls of the flask.

Conclusions agreed essentially with those from the previous experiment. In addition, it was found: 1) The exact viscosity grades and concentration of Methocel supplement were not critical for optimum stimulating effect, at least within the low ranges used. 2) The sizes of inoculum at the levels tested were adequate for the slow, sustained population increase of an agitated fluid-suspension culture through two tissue-culture generations.

Experiment 6.—This experiment was set up to corroborate the conclusions of previous experiments with respect to the effectiveness of: 1) medium NCTC 109 supplemented with Methocel, and 2) silicone-coated flasks for growing strain 2071 cells in large, agitated fluid-suspension cultures. Methocel supplement was used at only one concentration, 0.12 percent, and in one viscosity grade, 25 cps. Population trends, volume changes, and glucose utilization in one of these two cultures are shown in text-figure 1. Both cultures in this experiment were discontinued soon after the 17th day. Results from previous experiments were generally confirmed (table 1).

Experiment 7.—The objectives were: 1) to check the effectiveness of Methocel supplements at higher concentrations than had been used in



TEXT-FIGURE 1.—Total cell populations, cell concentrations, fluid volumes, and glucose concentrations in one agitated fluid-suspension culture in a 1½ liter flask. Each point on the population curves represents a nuclei count at a fluid change.

previous experiments, and 2) to furnish some cells grown under these conditions for analysis of their infrared spectrums—to be reported later. Short-necked, 500 ml flat-bottom boiling flasks of such size and shape that they could be centrifuged directly at each fluid change in a swinging-box type head (#925 for International #2 centrifuge) were used, according to the modification developed recently by Bakken, Evans, Stevenson, and Earle (*8*). This procedure avoids the necessity of pipette transfers: (*a*) of the cell suspension from the culture flask to centrifuge tubes and (*b*) of the centrifuged suspension back to the flask. Methocel supplemented the medium at two viscosity grades and at higher concentrations

than in the other experiments. Culture #1 had 10 cps Methocel at 1.02 percent final concentration, and culture #2 had 50 cps Methocel at 0.44 percent. The final viscosities of the 2 media were approximately the same.

The cells proliferated slowly but steadily. The conditions and the growth rates of the 2 cultures were closely similar to each other and were not significantly different from the other cultures receiving Methocel supplement and reported in this paper (table 1).

It was concluded that the lower viscosity grades of Methocel, over a wide range of final concentrations, were equally effective in protecting strain 2071 cells on a chemically defined medium.

DISCUSSION

Inoculum sizes, growth rates, and glucose utilization rates in 4 experiments have been averaged and are summarized in table 2. In all these, the logarithmic phase appeared to have begun immediately or within a few hours; the population curves have a nearly uniform slope from day zero to the end of the logarithmic phase, as illustrated by the proliferation curve in text-figure 1.

The highest inoculums (about 10^6/ml) were associated with the slowest logarithmic growth rate (expt. 5). The lower inoculums (4–5 $\times$ 10^5/ml) were associated with the intermediate growth rates expressed as 4 to 6.8 days' generation time.

Glucose utilization during the logarithmic phase by strain 2071 cells in various experiments on Methocel-supplemented protein-free, chemically defined medium NCTC 109 in agitated cultures (table 2) was at a slightly lower rate than that found previously for equivalent agitated cultures of strain 2071 cells or the parent clone 929 (L) cells in serum-supplemented medium NCTC 109 (*18*) The average rates presently reported were

TABLE 2.—Inoculum size, growth rate, and glucose utilization (averaged values) during the logarithmic phase of strain 2071 cells in suspension cultures in medium NCTC 109 supplemented with Methocel

		Logarithmic phase						Average glucose utilization	
		Inoculum population per culture ($\times$ 10^5)			End of phase population per culture ($\times$ 10^5)				
Expt. No.	Number of cultures	Total	Per ml	Duration (*days*)	Total	Per ml	Average generation time (*days*)	Concentration glucose in fresh media (*mg percent*)	Glucose used per million nuclei per day (*mg*)
4	8*	17. 2	0. 57	5	40. 3	1. 44	4. 05	100	Not determined
5	6	101. 2	1. 01	21	472. 2	1. 18	9. 45	100	0. 26
6	1	40. 4	0. 40	17	455. 3	1. 14	4. 86	260	0. 35
7	2	42. 6	0. 43	13	158. 7	0. 82	6. 85	100	0. 20

*Four treatments of 2 cultures each are represented in averages for experiment 4: C, D, E, F, listed in table 1.

from 0.20 to 0.35 mg glucose/10^6 cells/day, while the rates previously reported ranged from 0.25 to 0.50 mg glucose/10^6 cells/day. The lower rate of glucose utilization by these cells seemed related to their relatively slow proliferation rate. Also, the lessened glucose uptake could result from decreased permeability of the cell walls to which methylcellulose molecules may have bound.

Glucose utilization rates were significantly higher in experiments in which the glucose concentration in the fresh medium at each fluid change was 260 mg/100 ml than in those in which it was 100 mg/100 ml. Thus the presence of excess glucose in the medium resulted in higher glucose uptake by the cells. This trend agrees with earlier work with suspension cultures in this laboratory (*19*) and with studies of carbohydrate utilization in static cultures by Eagle and coworkers (*23*).

Certain inadequacies of the means used to protect the cells in suspension cultures are apparent. The growth cycles of all these cultures were somewhat abnormal. Proliferation in the logarithmic phase was generally at a slow rate; the phase was usually of long duration. In some cultures the transition from the logarithmic to the plateau phase was so gradual that it could not be located exactly.

It should be emphasized, however, that even this somewhat limited growth represents a major positive result, when it is contrasted with the immediate breakdown of the same cells in such cultures on a protein-free, chemically defined medium without methylcellulose supplement. Restrictions that were imposed on any protective agent used ruled out the protein hydrolysate such as has been used as a supplement to protective agents (*12–14*).

Katsuta and coworkers (*13*) used various high molecular-weight substances, "unrelated to cell nutrition," as supplements for protein-free media for strain L cells in stationary flasks. Their unsupplemented medium, however, differed from the protein-free, chemically defined medium used in the present study since it was protein-free but not chemically defined in that it contained lactalbumin hydrolysate. This compound is comprised of not only amino acids but also polypeptides in unknown proportions, sizes, and compositions, which may be protective in themselves. They found that L cells grew well, for apparently unlimited periods, on such medium supplemented with small amounts of PVP of known average molecular weights. With rat ascites cells (*12*, *14*) the substitution of either methylcellulose or PVP or dextran for 50 to 75 percent of the serum proteins gave optimum cell proliferation. In the absence of serum protein, the addition of these substances gave only a low rate of cell proliferation, though a small amount of lactalbumin hydrolysate was always present. They concluded that serum proteins are necessary for the cells physicochemically rather than nutritionally. However, it should be emphasized that their work was solely with *static* cultures, not with rapidly agitated cultures.

Phillips and Andrews (*15*) found that hydrophilic macromolecular substances, such as serum, gelatin, and 15 cps methylcellulose, protected

mammalian cells on balanced saline in static cultures, and suggested that the mechanism involved an increase in oncotic pressure, *i.e.*, colloid osmotic pressure. The culture conditions differed in major points from those in the present study: They studied cells only in *static* flasks and only for survival during 4 hours in the experimental media. Nevertheless, the possibility might be considered that in the present study methylcellulose protects the cells partly by means of such an increase in oncotic pressure. This possibility awaits further study.

No definite conclusions can presently be reached about partial substitution of methylcellulose for glucose, either in the foodstuffs requirements of these cultures or in the reducing sugar analyses of the spent fluids. Control analyses of various fresh Methocel solutions, whether in water, in Earle's saline, or in medium NCTC 109, have consistently shown that the presence of methylcellulose does not increase the reducing sugar values. This fact presumably indicates that reducing sugar analyses of the spent medium do not include significant amounts of either methylcellulose or hydrolytic products.

The anomalous effect of serum supplement on 2071 cells, which were on medium NCTC 109 in silicone-coated agitated flasks, was unexpected. Serum-supplemented medium was introduced in several experiments primarily as a positive control, but invariably resulted in disintegration of the cells after short periods while the comparable Methocel-supplemented cultures continued to proliferate. Previous experiments from this laboratory (*9*) with strain 2071 or closely related strain 2168 cells in agitated flasks not silicone-coated showed that serum-supplemented NCTC 109 was an adequate medium. Moreover, McQuilkin, Evans, and Earle (*4*) reported that strain 2071 cells in static cultures after 595 days on medium NCTC 109 continued to proliferate at a high rate even when serum was included with the medium. This finding has been confirmed more recently by McQuilkin (personal communication) for 2071 cells in static cultures, returned to 20 percent horse serum plus NCTC 109 after 4½ years on medium NCTC 109 alone. Thus the failure of 2071 cells on serum-supplemented NCTC 109 in silicone-coated agitated flasks must await further study. For the present, silicone appears beneficial in keeping cells off the glass on Methocel-supplemented medium NCTC 109.

The mechanism by which methylcellulose supplement to medium NCTC 109 exerts a protective effect on strain 2071 cells in agitated suspension cultures must also await further study. As a working hypothesis it may be proposed that 1) methylcellulose molecules attach to the cell wall to strengthen it physically against breakdown. The nature of the binding may be presumed to be nonionic, since the Methocels used for most of the experiments were nonionic cellulosic polymers in 1,4-glucosidic linkage with only methoxyl substituents; if some carboxyl groups are present, as Kuchler *et al.* (*11*) have suggested, the binding might be partly ionic. 2) Attached methylcellulose molecules might alter the cell wall so that permeability is greatly reduced. If so, highly diffusible essential materials fabricated within the cell might be retained within the cell, and thus

protect it. At the same time, the passage of essential molecules from the media into the cell might be hindered so as to slow down the metabolic activity and the proliferation rate.

In summary, there are three different agents in this milieu whose influences are not yet known: methylcellulose, silicone, and serum. The methylcellulose-silicone-NCTC 109 milieu is definitely advantageous for strain 2071 cells in the rapidly agitated fluid-suspension cultures, but it must be considered inadequate compared to the full potentialities that have already been achieved by cells of the same clone grown in medium NCTC 109 alone in static cultures.

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Control of Some Cell-Contact Reactions in Tissue Culture [1, 2]

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SUMMARY

The degree of contact inhibition, a contact property shown by normal but not by tumor cells, produced in chick-heart fibroblasts from 9-day-old embryos grown in a liquid medium of serum, embryo extract, and saline, decreases with increasing embryo-extract concentration and with increasing age of the culture. Contact inhibition is controlled in part by the nature of the culture medium, but its display is not directly dependent on the speed of movement of the cell, though it is suggested that both may have a common controlling mechanism. It is not related directly to the incidence of mitosis past or present, but depends partly on the density of cell population.—J. Nat. Cancer Inst. 26:253–268, 1961.

IT HAS been the purpose of this work to investigate the nature of contact inhibition as it is manifested in normal fibroblasts. Abercrombie (*1*) has suggested that the occurrence of this phenomenon may be one of the chief factors which prevents normal cells from becoming invasive, and has shown that invasive sarcoma cells lack this property. If two fibroblasts in tissue culture meet while moving on a plane surface, they tend to stop moving as soon as contact is made between them. This tendency to the cessation of movement on contact was described by Abercrombie and Heaysman (*2*) and named "contact inhibition." Should two cells, whose movement has been stopped by their contact, not be in contact with other cells, then they may move away from each other in any direction that does not immediately bring them into contact with cells. Obviously this phenomenon prevents the overlap of cells and tends to keep the outgrowth as a monolayer. Abercrombie (*1*) has discussed how contact inhibition would be expected to act in determining the form of the outgrowth in a tissue culture. However, previous work has not described whether it is an inherent and a permanent property of fibroblasts expressed under all living conditions, or to what other properties of the cell it is related. The present work

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attempts to answer these questions in part, to provide preliminary data for discussion of the mechanism of contact inhibition and for the consideration of how invasive cells differ from normal ones.

Pioneer experiments by Abercrombie, Curtis, and Karthauser (*3*) suggested that the application of medium containing high concentrations of embryo extract diminished the degree of contact inhibition. For this reason, media containing differing concentrations of extract have been applied to the cells. Moreover, the application of embryo extract allows a test of two possible mechanisms to account for the incidence of contact inhibition in a culture. High concentrations of extract in culture media are known to affect the incidence of mitosis and the speed of cell movement (*4*). Abercrombie (*1*) reports that cells in mitosis do not shown contact inhibition; obviously a decrease in contact inhibition might be caused by an increase in mitoses. In consequence the relation between mitoses and contact inhibition has been tested over a range of culture conditions. The second possible mechanism for the occurrence of contact inhibition is that a cell which stops movement on contact with another is not moving rapidly enough to have sufficient impetus to move across it. If this is so, speed of movement of cells should be correlated with the degree of overlap they show, overlap being inversely related to contact inhibition. Tests of this hypothesis have been made on cultures treated with differing concentrations of extract which can be expected to shown differing speeds of cell movement.

METHODS

Culture methods.—Hearts from 9-day-old chick embryos were explanted in small pieces in pairs on glass coverslips. They were grown in a liquid medium made from Pannett-Compton saline, serum from cocks, and extract from whole 9-day-old chick embryos. A standard (normal) medium was composed of 50 percent serum, 25 percent saline, and 25 percent extract, and was used in all cultures save those in which it is stated to the contrary. The embryo extract contained 50 percent Pannett-Compton saline. All cultures were started in this medium, the extract being prepared and the medium mixed a few hours before the explants were made. The cultures were grown at 37 to 38° C as standing drops for 19 hours and thereafter as hanging drops. They may be divided into three series:

1) The culture medium was replaced after 19 hours by a fresh medium, and the cultures were fixed after 24 hours. The replacement media were mixed shortly before application, the same extract used as in preparing the original medium. The extract had been stored at 0° C overnight. In this series three different replacement media were used. The first contained twice the normal concentration of extract, the second was identical with the standard medium, and the third contained half the normal concentration of extract. These cultures will be referred to as

the 2EE, 1EE (normal), and ½EE groups. All these media contained 50 percent serum, but since the extract concentration in the media was altered by varying the proportions of extract to saline, they also varied in their saline-solids content to some degree, for extract may contain as little as 50 percent by volume of saline itself. This method of preparation of the media meant that the cultures were subjected to two variables at once, and in consequence a subsidiary group of cultures was treated with media containing 133, 100, and 66 percent of the normal amount of saline solids, but not varying in any other component. These are called 133, 100, and 66 percent groups hereafter and represent greater variations in saline-solids concentration than might occur in the media of differing extract concentration.

2) No replacement of the culture medium was made. Cultures were fixed at 24, 48, 66, and 72 hours after explantation. Hereafter these are referred to as 24U, 48U, 66U, and 72U, respectively.

3) These cultures were grown for 66 hours in the original, unchanged medium, which was then replaced with normal medium, freshly mixed and containing either the same extract as had been used for preparation of the original medium stored at 0° C or extract prepared an hour previously from whole 9-day-old chick embryos. These cultures are referred to as 66A and 66N, respectively. They were fixed at 72 hours of age. All cultures were fixed in formol-saline and stained with Mallory's hematoxylin.

Measurement of the speed of cell movement.—The rate of movement of fibroblasts was measured by time-lapse cinematophotography of the cultures. Four cultures from each of the 4 groups (all groups of Series 1 and 24U) fixed at 24 hours were filmed from 20 to 24 hours of age. The films were made on Plus-X (Kodak) with a phase-contrast system for producing the image (10× objective, N.A. 0.4, and projecting eyepiece). A Sylvania C100 zircon arc lamp run at 7.0 amps was used for illumination, with a Chance ON 22 heat filter, neutral density filters of value 1.1, and a Wratten 58 filter (green) which controlled the exposure. A light shutter was placed between the lamp and the microscope to diminish the period of illumination of the culture; this shutter opened for a half second for each exposure, during which the frame was exposed for 0.025 seconds. Films made with either a 30- or 50-second interval between frames were analyzed by projection. The position of 20 cells on the screen was marked at frame intervals corresponding to 15 minutes. Distances between the positions of the cells were measured every 15 minutes and converted to true distances with the aid of a stage micrometer image which had been photographed at the end of each film. The results are expressed in mean distances traveled per 20 cells in micra per hour. Five measurements were made on each film on the last 5 successive 15-minute periods. All filming was done inside a room kept at 36 to 37° C.

Measurement of contact inhibition.—Virtually the same method as that used by Abercrombie and Heaysman (*2*) was applied. Counts of observed overlaps of nuclei (these counts are referred to as "O") and total numbers

of cells were made in all cultures in one region measuring 140 $\mu \times 140$ μ in the center of the possible overlap. This region, hereafter called "central" (*see* text-fig. 1) is that in which cells from either explant would be found if there were no impediment to their motion outward from the explants. Since the outgrowth enlarges throughout the life of the culture, the older the culture is to be at the time of fixation, the farther apart the explants can be placed originally and yet allow a region in which overlap is possible at the time of fixation. There is a possibility of collagen formation after 40 hours of culture (*5*), which might alter the relations of cells to each other. Collagen formation spreads outward from the explants. In consequence, cultures to be fixed after longer culture periods were made with their explants placed farther apart, so that the region of overlap was less likely to contain collagen fibers. This precaution allows measurement in areas where cells from either outgrowth have only recently come in contact. Thereby areas, which might show conditions of overlap appropriate to a much earlier stage in the history of the culture, are excluded.

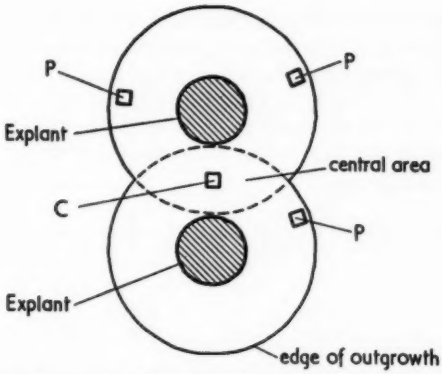
In calculating the overlap, E, expected in the absence of contact inhibition, I used the following formula:

$$E = \frac{\pi a.b.\ n\ (n-1)}{2A}$$

where n is the number of cells counted in the area A, and a and b are the mean values for the length of the 2 axes of the ellipse that represents nuclear shape. Abercrombie and Heaysman (*2*) used a formula for E which is identical with the above, except that they treated the nuclei as being circular in shape, of radius r. Thus they wrote the expression $\pi a.b.$ as $\pi(2r)^2$. Treating the nuclei as ellipses is a closer approach to the actual nuclear form. When values of E were calculated for each culture, mean values of a and b were obtained from the same region as were the observed overlaps; the means are of samples of 10 paired measurements of nuclei. These values are included in the values of E calculated.

The ratio $\frac{\text{Actual overlap (O)}}{\text{Expected overlap (E)}}$, hereafter called the overlap ratio (O/E), has been used to express the degree of contact inhibition shown by cultures. If a value of O/E = 1.0 is found, there is no contact inhibition; likewise a value of 0.1 would indicate far fewer overlaps than would be expected on random grounds, which implies a high degree of contact inhibition.

Enumeration of mitoses.—The number of mitoses and cells in the same field as had been counted for overlaps and those in two similar adjacent fields were recorded. Each field measured 140 $\mu \times 140$ μ. The total number of cells and mitoses for all three areas are hereafter called "central cells and mitoses." Likewise three regions at the periphery of the



TEXT-FIGURE 1.—Arrangement of explants (cross-hatched) and outgrowths at time of fixation. Area of possible overlap, called "central" area, lies inside dotted lines, and block, C, represents possible central counting area. Three blocks marked P represent peripheral counting areas.

outgrowth (*see* text-fig. 1), each 140 $\mu \times 140\ \mu$, were counted for cells and mitoses; these are referred to as "peripheral."

Statistical methods.—In general these are standard methods. Mitotic counts are Poisson-distributed, and in consequence results are given as means alone. In order that tests of significance may be made on these results, the transformation recommended by Snedecor (*6*) of taking $\sqrt{\text{count} + 0.5}$ was used. Tests of significant difference in the means from distributions of different size were carried out according to the method of Cochran and Cox, given by Snedecor (*6*). In all tests of significance, results at or below the 1 percent probability level are considered to be highly significant, and those between 2.5 and 1 percent levels moderately significant. Results with a probability greater than 2.5 percent are considered to be nonsignificant.

RESULTS

Results are arranged in terms of the three series of experiments. A full set of experimental measurements was made for the first series, but, for the other two, only overlap values and mitotic counts have been made. In all cases n refers to the number of measurements made, and, except for the data on speed of cell movement, to the number of separate cultures examined; SE refers to the standard error, r to the correlation coefficient, P to the probability in statistical tests, and d.f. to the degrees of freedom in such a test.

Series 1: Effect of Different Concentrations of Extract—Cultures Fixed at 24 Hours

The overlap ratio (contact inhibition) data for this series are given in table 1. The mean overlap ratio in the 2EE group is significantly greater

than in the group treated with normal medium—the 1EE group ($t = 6.67$, $P << 0.001$, d.f. 48). Likewise it is greater in the normal (1EE) group than in the ½EE group ($t = 6.19$, $P << 0.001$, d.f. 48). Comparison of the ½EE group and the 24U group is made by the method of Cochran and Cox (difference of means/standard error of differences = 2.80, 1% level = 2.816), which indicates a significant difference between the means of these two groups.

TABLE 1.—Overlap ratios (O/E) and speed of cell movement for Series-1 cultures

Overlap					
Group	n*	Mean population density	Standard error	Mean O/E	Standard error
2EE	25	65. 1	2. 98	0. 786	0. 0376
1EE	25	60. 1	3. 00	0. 470	0. 0298
½EE	25	59. 8	2. 84	0. 226	0. 0256
24U	20	45. 3	0. 64	0. 139	0. 0179

Speed of movement			
Group	n	Mean speed μ/hour	Standard error
2EE	20	39. 8	3. 94
1EE	20	61. 3	2. 37
½EE	20	48. 2	2. 82
24U	20	50. 6	2. 98

*Sample size.

The data on the speed of cell movement are also given in table 1. The mean speed of movement in normal medium is significantly greater than in the 2EE group ($t = 4.63$, $P < 0.001$, d.f. 38) and greater than in the ½E group ($t = 2.42$, $P = 0.02$, d.f. 38). Likewise, cells move more rapidly in the 1EE group than in the 24U group ($t = 2.80$, $P < 0.01$, d.f. 38). However, speed of movement in the ½EE group is not significantly different from that in the 24U group ($t = 0.50$, $P > 0.2$, d.f. 38).

These results show that the effect of increasing concentration of embryo extract in the medium is a continuous rise in the degree of overlap shown by the cells, but their rate of movement only increases over the lower end of the range of overlap increment with increasing extract concentration. Speed decreases again as the overlap ratio increases still further. Such a result shows that the rate of cell movement cannot alone account for the degree of contact inhibition shown by the cells, according to the theory that increased motility allows increased overlap.

In assessing the nature of mitosis in the cultures of this series (*see* table 2) I have calculated the correlation coefficient for regression between number of mitoses and cell density to discover whether mitoses should be presented either as number per unit area or as number per 100 cells. Since significant correlation between cell number and mitoses is found in one case alone, comparison between the incidence of mitosis in the various groups has been made in terms of mitoses per unit area and not

per unit cell population. A possible reason for the failure to find a correlation between numbers of mitoses and cells is that the areas sampled are sufficiently small for a very great variance in mitoses. It is also possible that mitoses tend to occur locally in clumps.

TABLE 2.—Mitoses in Series-1 cultures, centrally and peripherally

Group	n	Mean population density	Standard error	Mean mitoses	Correlation coefficient
Centrally					
2EE	20	109.7	5.51	3.37	0.061 $P>0.2$
1EE	20	91.0	5.72	2.61	0.656 $P<0.01$
½EE	20	104.1	5.43	2.82	−0.048 $P>0.2$
24U	20	104.2	2.42	3.26	−0.152 $P>0.2$
Peripherally					
2EE	20	66.2	2.78	2.89	0.375 $P>0.1$
1EE	20	63.3	2.69	1.84	0.315 $P>0.1$
½EE	20	65.2	3.16	2.04	0.104 $P>0.2$
24U	20	69.0	3.26	2.23	0.261 $P>0.1$

Centrally there is no significant difference in mitotic numbers between the 2EE group and the 1EE group ($t = 0.97$, $P>0.2$, d.f. 38), or between 1EE and ½EE groups ($t = 0.32$, $P>0.2$, d.f. 38), or between the ½EE group and the unchanged group, 24U ($t = 0.73$, $P>0.2$, d.f. 38). As for the number of mitoses at the periphery of the outgrowths, there is no significant difference between the 2EE and 1EE groups ($t = 1.92$, $P>0.05$, d.f. 38), or between 1EE and ½EE groups ($t = 0.39$, $P> 0.5$, d.f. 38), or again between the ½EE group and 24U ($t = 0.39$, $P>0.5$, d.f. 38).

These results show that there is no difference in the number of mitoses found either in the area in which contact inhibition is measured (centrally), or in the periphery of the outgrowth, under a series of treatments which cause significant differences in the incidence of contact inhibition.

Does a correlation between mitoses and the degree of overlap exist inside each group? To test this possibility, I calculated the regression of overlap ratio on central mitoses, and found correlation coefficients as follows:

$$2EE : r = 0.369,\ P>0.1 \quad 1EE : r = -0.495,\ P = 0.027$$

$$½EE : r = 0.270,\ P>0.1 \quad 24U : r = 0.015,\ P>0.1$$

Three of these values show no significant correlation, the fourth is a borderline case, but the negative sign of r argues against the thesis that the more mitoses the greater the overlap ratio. These results show that mitoses actually in progress at the time of fixation do not account for the degree of contact inhibition shown by a culture. Later these and other results will be used to show that the mitotic history of a culture over a long period is such that the incidence of mitoses cannot account for the degree of overlap developed.

It is possible that the degree of contact inhibition may alter as the

population rises. To investigate this possibility further, I calculated the correlation between the O/E ratio and cell population for each of the four groups (table 3).

TABLE 3.—Linear regression of overlap ratio (O/E) on population in Series 1

Group	Whole population		Upper portion		Lower portion	
	n	Correlation coefficient	n	Correlation coefficient	n	Correlation coefficient
2EE	25	0.534 $P = 0.01$	—	Not calculated	—	Not calculated
1EE	25	0.254 $P > 0.2$	12	0.088 $P > 0.2$	12	0.644 $P = 0.025$
½EE	25	0.432 $P = 0.04$	12	0.073 $P > 0.2$	12	0.702 $P = 0.012$
24U	20	0.160 $P > 0.2$	—	Not calculated	—	Not calculated

The relation between the overlap ratio and population is highly significant for the 2EE group and slightly significant for the ½EE group, but is not significant in other cases. If the correlation coefficients between population and O/E ratio are calculated separately for lower and upper ranges of the population (table 3), it appears that there is a significant rise of the overlap ratio with population in the lower ranges for the 1EE and ½EE groups. This rise extends over the whole range for the 2EE group.

Since the increment of overlap ratio with population, together with population differences, might alone account for the differences found between the groups in overlap ratio, the overlap comparisons have been re-examined. First, when the means of O/E ratios for upper and lower groups in the same treatment are compared, no significant difference is found for the 1EE group ($t = 0.88$, d.f. 22, $P > 0.2$). With the ½EE group a slightly significant result is found ($t = 2.15$, $P = 0.04$, d.f. 22). Comparisons between treatments for upper or lower parts of the 2EE and 1EE groups show significant differences in their means (for upper groups, $t = 7.81$, $P < 0.001$, d.f. 22; for lower groups, $t = 3.03$, $P < 0.01$, d.f. 22). When 1EE and ½EE groups are compared, both upper ($t = 5.94$, $P < 0.001$, d.f. 22) and lower ($t = 3.49$, $P < 0.001$, d.f. 22) ranges are significantly different. To establish these differences the more certainly, one should point out that the samples from which they are drawn are not significantly different in their population means. For upper groups, comparing population means between 2EE and 1EE ($t = 1.23$, $P > 0.1$, d.f. 22) and between 1EE and ½EE ($t = 0.27$, $P > 0.2$, d.f. 22), I found no difference. For lower groups, population means between 2EE and 1EE ($t = 1.75$, $P > 0.5$, d.f. 22) and between 1EE and ½EE ($t = 0.55$, $P > 0.2$, d.f. 22), I found no difference. The variance of the population data of these groups shows no significant differences at the 1 percent level, where $z = 0.750$ for n_1 and $n_2 = 11$. In the lower groups for 2EE and 1EE, $z = 0.692$, and for 1EE and ½EE, $z = 0.356$, while for upper groups, $z = 0.416$ for 2EE and 1EE and $z = 0.099$ for 1EE and ½EE. Thus there are no differences in population means or variances between the groups. It can be concluded that, despite the increment in overlap ratio with population over all or part of

the population range, all 3 groups have significantly different values for this ratio, at any point in the population range.

In summary of these results it can be seen that, if the culture medium is replaced at 19 hours of age by media containing varying amounts of embryo extract, the following occur 5 hours later: (*a*) The greater the concentration of extract, the lower the degree of contact inhibition, but no difference in the incidence of mitoses appears between the various treatments either centrally or peripherally. (*b*) Cell movement is most rapid in cultures with normal extract concentration, but slower in those with more or less extract. (*c*) Contact inhibition diminishes with population increase over the lower parts of the population range. Speed of cell movement in those cultures with unchanged media fixed at the same age (24U group) resembles that in the ½EE group.

Since media containing differing amounts of extract also varied in their saline contents, a small series of cultures containing 133, 100, or 66 percent of their normal saline solids, but no other differences, were measured for their O/E ratios. The results are given in table 4. There is no significant difference in the means of the O/E ratios between the 133 and 100 percent groups, or between the 100 and 66 percent, or between the 133 and 66 percent ($t = 0.089$, d.f. 9). Moreover, there is no difference between the 100 percent group and the 1EE group (method of Cochran and Cox). These results show that changes in the saline solid concentration in the groups treated with different extract concentration cannot in themselves be responsible for the different degrees of overlap, and that the components extracted from the embryos must be active in affecting contact inhibition.

TABLE 4.—Overlap ratio (O/E) in cultures with differing saline concentrations

Group (*percent*)	*n*	Mean population density	Standard error	Mean O/E	Standard error	Values of *t* between groups
133	10	53. 4	2. 45	0. 432	0. 040	0. 293
100	10	52. 6	2. 99	0. 449	0. 046	0. 381
66	10	50. 0	1. 47	0. 506	0. 039	
		(for d.f. = 9, $t = 3.25$, at $P = 0.01$)				

Series 2: Cultures Grown in Unchanged Media for Varying Times

This series comprises cultures grown for 24, 48, 66, and 72 hours without change of medium and hereafter are called 24U, 48U, 66U, and 72U, respectively. Results for 24U have been given with Series 1 and are not repeated here. Data on overlap ratios and mitoses are given in table 5.

A significant difference in the mean O/E ratio is found between 24U and 48U groups ($t = 11.82$) and between 48U and 66U groups, but not between the 66U and 72U groups. It appears that contact inhibition diminishes as a culture ages with its medium. As with Series 1, the correlation for linear regression between mitoses and cell number has been

calculated (table 5). In only one case is there a significant correlation, for the 48U peripheral group. Because of this general lack of correlation, mitoses have been compared per unit area. If the 1EE and 48U groups are compared, a significant difference is found for central, but not for peripheral mitoses (central pairs: $t = 3.48$, peripheral pairs: $t = 2.18$, $P > 0.025$, d.f. 38). There are significantly fewer mitoses at 48 hours than at 24 (central pairs: $t = 5.27$, peripheral pairs: $t = 3.32$, d.f. 38), and likewise again at 66 hours than at 48 (central pairs: $t = 2.78$, peripheral pairs: $t = 2.59$, $P = 0.015$, d.f. 38). But there is no difference between 66U and 72U (central pairs: $t = 0.66$, peripheral pairs: $t = 1.20$, d.f. 38).

TABLE 5.—Overlap ratio (O/E) and mitoses in Series-2 cultures

Overlap ratio

Group	n	Mean population density	Standard error	Mean O/E	Standard error	Values of t between groups
48U	20	52.6	1.81	0.478	0.018	4.411
66U	20	54.1	2.06	0.734	0.053	0.313
72U	20	52.1	1.98	0.755	0.040	
			(for d.f. = 19, $t = 2.861$ for $P = 0.01$)			

Central mitoses

Group	n	Mean population density	Standard error	Mean mitoses	Correlation coefficient
48U	20	125.0	3.87	0.87	0.309
66U	20	124.1	3.88	0.17	0.116
72U	20	114.0	3.02	0.28	0.205
			(for d.f. = 18, $r = 0.561$ for $P = 0.01$)		

Peripheral mitoses

Group	n	Mean population density	Standard error	Mean mitoses	Correlation coefficient
48U	20	54.1	2.73	0.84	0.566
66U	20	57.0	2.72	0.19	0.075
72U	20	58.1	2.86	0.43	0.009

In this series there is a decrease in the incidence of mitoses, both central and peripheral, from 24 to 66 hours of age. This is accompanied by a decrease in the degree of contact inhibition. Tests for the correlation of mitotic incidence and the degree of overlap inside each group were done by calculation of the correlation coefficient for linear regression r. Only in the 48U group is the value of r significant of a correlation ($r = 0.544$, $P = 0.012$, d.f. 18). For the 24U group $r = 0.015$, $P > 0.2$; for the

66U group $r = 0.015$, $P > 0.2$; and for the 72U group $r = 0.123$, $P > 0.2$; none of these values is significant at d.f. 18.

The linear regression of overlap ratio on cell population was calculated, both for the whole of each population and for the lower half, to discover the existence of any such relation (table 6). None of these values is significant of a correlation. Thus, although there is no difference in the overlap ratio between the 1EE and 48U groups (with the method of Cochran and Cox), the first shows a correlation between population and overlap ratio over part of its population range, whereas the 48U group does not. It begins to seem that this relation between overlap ratio and population appears only in cultures which have recently received fresh medium, but further evidence on this point appears in the results from Series 3.

TABLE 6.—Linear regression of overlap ratio (O/E) on population in Series 2*

	Whole population		Lower portion	
Group	n	Correlation coefficient	n	Correlation coefficient
48U	20	0.289	12	−0.570
66U	20	0.179	12	−0.281
72U	20	−0.342	12	0.421

* $r = 0.561$ for $P = 0.01$, d.f. = 18, and $r = 0.708$ for $P = 0.01$, d.f. = 10.

Series 3: Cultures With Medium Replaced at 66 Hours

Overlap ratios and mitotic counts are given in table 7. There is significantly less contact inhibition in the group treated with aged medium than in the other. Comparing these results with the two appropriate control groups, 66U and 72U, I found that there is significantly more overlap in the 66U group than in the 66N group ($t = 9.97$, $P < 0.001$, d.f. 19). Again, there is less overlap in the 66N group than in the 72U group ($t = 13.21$, $P < 0.001$, d.f. 19). However, there is no difference between the 66A and the 66U groups ($t = 0.56$, $P > 0.1$, d.f. 19), or between the 66A and 72U groups ($t = 1.03$, $P > 0.1$, d.f. 19). From these results it appears that the application of medium containing freshly prepared extract will increase the degree of contact inhibition in aged cultures, whereas medium containing aged extract (66A group) does not alter the degree of contact inhibition.

If mitoses are compared, it can be seen that there is no correlation between them and cell numbers in these samples. There is no difference in the incidence of mitoses between these two groups, either centrally ($t = 1.38$, $P > 0.1$, d.f. 38) or peripherally ($t = 0.29$, $P > 0.1$, d.f. 38). However, there are more central mitoses in the 66N group than in the 72U group ($t = 3.74$, $P < 0.001$, d.f. 38), though not peripherally ($t = 0.08$, $P > 0.1$, d.f. 38). In the aged group 66A there is no difference either centrally ($t = 1.60$, $P > 0.1$, d.f. 38) or peripherally ($t = 0.46$, $P > 0.1$, d.f. 38) between it and the 72U group. Again there is no significant

TABLE 7.—Overlap ratio (O/E) and mitoses in Series-3 cultures

Overlap ratio						
Group	n	Mean population density	Standard error	Mean O/E	Standard error	Value of t between groups
66N	20	46. 1	1. 09	0. 182	0. 015	11. 925
66A	20	47. 9	1. 79	0. 696	0. 040	
Central mitoses						
Group	n	Mean population density	Standard error	Mean mitoses	Correlation coefficient	
66N	20	108. 3	2. 85	1. 21	0. 230	
66A	20	113. 0	5. 22	0. 73	0. 371	
Peripheral mitoses						
Group	n	Mean population density	Standard error	Mean mitoses	Correlation coefficient	
66N	20	60. 1	3. 27	0. 62	0. 394	
66A	20	58. 4	5. 02	0. 55	0. 024	
(for r = 0.561, P = 0.01 at d.f. = 18)						

correlation between mitoses and overlap in these two groups, for 66N $r = -0.130$, and for 66A $r = 0.192$, d.f. 18.

Earlier results from the second series showed a progressive increase in the overlap ratio with culture age, accompanied by a fall in the incidence of mitoses. This argues strongly against the idea that the cells in mitosis, or recently so, account for the greater part of the overlaps found. However, once cells have overlapped, they may tend to remain in overlap. In consequence, the steadily increasing overlap ratio as cultures age might be no more than a reflection of the fact that the sum total of past mitoses increases. Evidence from the 66N group is strongly against this idea. In this group the overlap ratio is greatly reduced in comparison with that in either the 66U or 72U groups, whereas the numbers of mitoses have remained the same or increased, and these changes have occurred after 6 hours, whereas the cultures have had a previous life eleven times as long. The incidence of mitoses does not account for the degree of overlap in any of these cultures.

The linear regression of the overlap ratio on cell population was calculated for the whole of each population and for the lower 12 values of population.

For 66N group, $n = 20$, $r = 0.486$; for lower portion, $n = 12$, $r = 0.719$.

For 66A group, $n = 20$, $r = 0.370$; for lower portion, $n = 12$, $r = 0.400$.

Since $r = 0.708$ for $P = 0.01$ at d.f. 10, it is plain that only the correlation for the lower portion of the 66N group is significant. A subsidiary experiment gives further information on this point. A group of 20 cultures was treated like the 1EE group save that they were fixed at 36 hours of age. In this group, mean cell number = 49.5, SE = 1.40, mean O/E ratio = 0.247, SE = 0.023, correlation coefficient for population and overlap, $r = -0.129$ for the whole population, while for the lower 12 values, $r = -0.298$. The correlation between overlap ratios and population was found only in cultures which were fixed 6 hours after receiving new medium containing fairly fresh extract, and was not found some 16 hours after the new medium was given. This series of experiments shows that the display of contact inhibition in a culture is still alterable by medium changes after prolonged culture.

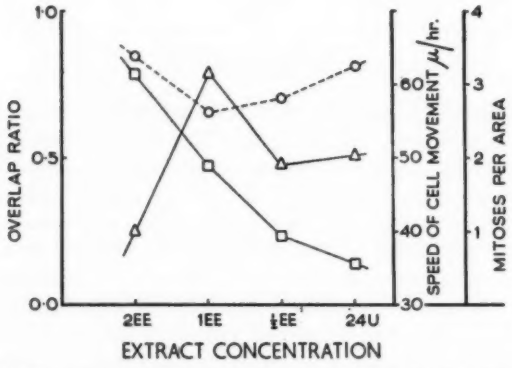
DISCUSSION

Normal fibroblasts tend to show contact inhibition toward other normal cells, whereas invasive cells do not. The present work has been an investigation of the nature of contact inhibition in normal fibroblasts to discover more precisely the difference between normal and invasive cells. The results are of significance in four respects: 1) Contact inhibition is not a property controlled by the cell alone but depends to a considerable degree on the conditions of culture for its quantitative expression. 2) The degree of contact inhibition is not directly dependent on the speed of movement of a cell; the success or failure of the overlap of cells is not a consequence of their having or failing to have sufficient speed of movement. 3) Contact inhibition is not accounted for by the extent of mitosis in a culture, and thus is not directly related to the rate of increase of cell population. 4) The degree of contact inhibition shown by a cell depends to a certain extent on the density of population in its locality, though this effect operates only at low population densities.

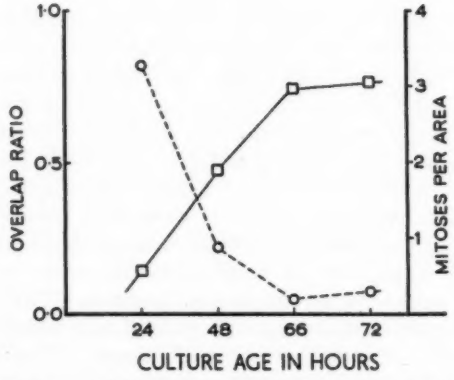
The main results of the experiments are diagrammatically summarized in text-figures 2 and 3. The experiments show that the degree of contact inhibition (the lack of overlap of the cells) is closely dependent on the conditions of culture, and that in any given conditions it can be measured quantitatively by the overlap ratio. It is affected by the concentration of embryo extract in the medium, at least early in the life of the culture. The degree of contact inhibition shown by the cells also decreases as the culture ages, and this occurs, probably because of changes in the nature of the culture medium, for the following reason: Application of medium containing fresh extract at 66 hours produces more contact inhibition than occurs in cultures with unreplaced medium. This shows that the display of contact inhibition can still respond to a change in the nature of the culture medium after this length of culture. Therefore the display of contact inhibition is a reversible property at any time during the culture. Although the results show that the nature of the culture medium

is of great importance in the control of contact inhibition, it is impossible at present to exclude the hypothesis that changes in the cells themselves as they age affect the display of contact inhibition. At present there are no pairs of experiments done at different culture ages with the use of treatment with medium of identical properties so that the degree of contact inhibition in each may be matched, which would permit this question to be settled. If the presence or absence of contact inhibition is a factor which affects the invasiveness of cells, these experiments draw attention to the fact that alterations in the milieu of cells may alter their contact properties sufficiently to allow invasion.

The concentration of embryo extract in the culture medium affects the speed of cell movement. However, the relation is not a linear one,



TEXT-FIGURE 2.—Summary of data on overlap ratio (□ points), mitoses (○ points), and speed of cell movement (△ points) as mean values, for the effect of extract concentration. Extract concentration is greatest at 2EE, least at ½EE; 24U is a comparative point (*see* text in "Results").



TEXT-FIGURE 3.—Summary of data on overlap ratio (□ points), and mitoses (○ points) as mean values, for the effect of culture age.

for the most rapid movement occurs neither with a high nor low concentration, but with an intermediate one. This result might appear to be in disagreement with that of Willmer and Jacoby (*4*), who found that increasing extract concentrations stimulated movement. But their work was done with plasma clot and not liquid cultures; it is also possible that their extract was weaker than that used in the present work. The results suggest that, with additional measurements, a plot of speed of movement against degree of contact inhibition would give a humped curve, the maximum value of speed of movement corresponding with a moderate degree of contact inhibition. With less contact inhibition, *i.e.*, more overlap of the cells than at this maximum, the speed of movement of the cells falls off. The success of overlap is not due to the high speed of approach of the cells which allows their impetus to carry them over each other. Less overlap is obtained with the highest speeds, and in consequence this simple hypothetical mechanism for contact inhibition must be rejected. However, the curvilinear relation between movement and contact inhibition suggests that there may be some common mechanism controlling both phenomena. Accepting the hypothesis that cellular adhesiveness acts in producing contact inhibition (*1*), Curtis (*7*) suggested that this relation might exist. Speed of movement will be maximal for a moderate degree of adhesion, because greater adhesion slows down movement by friction, and less adhesion prevents movement because of the lack of purchase between cell and substrate.

The correlation between contact inhibition and the cell population density occurs when the density is low. The correlation does not exist at and above a certain population density in most groups. This limit can be used as evidence that contact inhibition is partly controlled by the number of contacts a cell has with neighboring cells: In the lower ranges of population density, contact number will increase more rapidly with population density than in the higher ranges, and there is of course a limit to the number of contacts that a given cell can receive. Curtis (*7*), in discussing the role of contacts between cells in the control of cell surface properties, suggested that at a contact between two fibroblasts there is an expansion of the surfaces relative to those portions not involved in the contact. Since adhesiveness is thought to increase with surface expansion (*7*) the region of contact becomes more adhesive than it was formerly, and this accounts for the contact inhibition of the cells by opposing their movement. Obviously, the more contacts a given cell develops, the less this difference in expansion between one part of the cell surface and another can appear, because the expansions and compressions due to each contact tend to balance. In consequence, contact inhibition will tend to lessen with increasing contact until it becomes impossible to get more cells in contact with a given cell. Population density and contact number are obviously closely related, with a limit to contact number, and the correlation of the first with contact inhibition and a limit have, of course, been found, which together suggest that contact inhibition is partly controlled by the number of contacts a cell has.

Nevertheless, at present other mechanisms for this dependence of contact inhibition on population density cannot be excluded.

Although this work is but a preliminary investigation, it shows that contact inhibition can be defined as a cell property controlled by the factors in the culture medium, such as the embryo extract content, and probably by the consequences of aging of the medium in contact with the cells. It is independent of mitosis, that is, of the direct effect of the rate of cell population increase, but is dependent to some degree on the local density of population probably due to the area of contact a cell has with others. Although contact inhibition is not caused by insufficient speed of cell movement, it seems probable that movement and the inhibition have a common controlling mechanism.

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Announcement

Manuscripts Approved for JNCI, November 15 to December 15, 1960

"Effect of foster-nursing on the morphology of the lymphatic tissue of mice," by S. Albert and Eileen Podolak.

"A transplantable bile-secreting hepatocellular carcinoma in the rat," by Melvin D. Reuber.

"Electron microscopy of tissue of the mammary glands and tumors in old mice with special reference to mitochondrial size," by J. C. H. de Man and Th. G. van Rijssel.

"A spontaneous tumor of the hamster with contagious manifestations," by Darlene C. Brindley and William G. Banfield.

ERRATA

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In the articles "Some Peculiar Biological and Biochemical Properties of a Mouse Hepatoma Induced by Chyrosidin. I, II, and III" by Zygmunt Albert and Marian Orłowski, chrysoidin should be spelled chrysoidine. It was incorrectly referred to as 2,4′-azodianiline instead of 2,4-diaminoazobenzene *or* 4-phenylazo-*m*-phenylenediamine.

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